University of Alberta

Regulation of Cl^-/HCO_3^- Anion Exchange Proteins

By

Deborah Ann Sterling ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Faculty of Graduate Studies and Research

The undersigned certify that they have read and recommended for acceptance to the Faculty of Graduate Studies and Research a thesis entitled **'Regulation of Cl^-/HCO_3^- Anion Exchange Proteins'.** Deborah Ann Sterling has submitted this thesis in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abstract

Plasma membrane Cl^-/HCO_3^- anion-exchange (AE) proteins mediate the electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane and thus contribute to the regulation of intracellular pH (pH_i), cell volume and concentrations of Cl^- and HCO_3^-. Many cellular processes are affected by changes in pH_i. Therefore, tight regulation of pH_i is essential. Cardiovascular diseases that clearly involve Cl^-/HCO_3^- exchange activity include ischemic injury and cardiac hypertrophy. An understanding of the role of AE proteins in regulation of pH_i is therefore very significant, especially with regard to AE transport activity in the heart.

This thesis focuses on the regulation of AE proteins by pH and carbonic anhydrases. Simultaneous changes of both pH_i and extracellular pH (pH_o) mimic ischaemic conditions. Results indicated that, under these conditions, AE2 transport activity was steeply inhibited by acid pH_i, whereas AE1 and AE3 were essentially insensitive to pH_i changes between pH 6.0-9.0.

The cytoplasmic carboxyl-terminal domain of AE1 contains a binding site for carbonic anhydrase II (CAII). Inhibition of AE transport activity by mutation of the CAII binding site, incubation with the CA inhibitor, acetazolamide and over-expression of the dominant negative, inactive V143Y CAII mutant indicated that binding of CAII accelerates AE1-, AE2- and AE3-mediated bicarbonate transport, without effect on the Cl^-/HCO_3^- transport protein, DRA. In the presence of V143Y CAII, expression of the extracellularly anchored CAIV restored full functional activity to AE1, AE2 and AE3. Sucrose gradient ultracentrifugation, gel overlay assays and GST pull-down assays revealed that

AE proteins physically interact with CAIV on the fourth extracellular loop of AE1.

Since AE1 and AE3 function under conditions that mimic ischaemia, we conclude that AE1 and AE3 can contribute to pH_i recovery after cellular-acid loading. We also conclude that CAII and CAIV physically and functionally interact with the AE proteins, but not DRA, to accelerate AE-mediated Cl^-/HCO_3^- anion exchange. The AE/CAII/CAIV complex forms a transport metabolon, a membrane protein complex involved in the acceleration and regulation of bicarbonate metabolism and transport.

Acknowledgements

So here comes the hard part, not that thanking people is hard, it's making sure that I've remembered everyone that poses the challenge. Let's start with Joe. I'm indebted to Joe for taking a chance and letting me come to his lab to study. I think the journey has been almost as enlightening and interesting for him as it has been for me. Joe taught me numerous skills that apply not only to science, but extend far beyond. He has taught me to question everything, until it has been shown conclusively that there is no alternative. Controls, controls, controls, a valuable lesson. He has strongly encouraged me to talk publicly about my findings and much as I didn't always appreciate it at the time, I realise now what a valuable skill communication is to have, particularly among scientists. Finally I'd like to thank him for being supportive of my future career choice, I fear another supervisor may not have appreciated my decision to leave research as favourably as Joe has and I appreciate his understanding.

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Now to the members of my both past and present. I'd like to thank Xia-bo Tang for teaching me many fundamental skills in the laboratory and to Jocelyne Fujinaga I'd like to extend my appreciation again for basic skills, but in addition for helping me develop a thick skin. It has come in handy many times. I'd also like to mention Andy Taylor who planted the intellectual property law seed in my head a couple of years ago. I'd like to thank him for pointing out that in some rare cases you can do science and still make money and for pointing me in the direction of the more comfortable lifestyle I am about to embark on. As for present members, Bernardo Alvarez generously donated two GST-fusion proteins he had prepared to me and for that I am grateful. Nathan Brown was a summer student who worked with me for a few months and helped collect some of the DRA transport assays. Thanks for giving me a hand Nathan. I'd also like to give an honourable mention to both Bernardo's wife Veronica and to another lab-mate, Quan-sheng. Their contributions to our lab pot-lucks have been outstanding. In particular to Veronica, thank you for awesome empenadas and alfa hores and to Quan-sheng, your secret recipe amazing chicken that to date cannot be reproduced by anyone else in the lab - and believe me we've tried.

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So now, I'm off to bigger and better things or maybe just different things, who knows? Thanks for the memories la la la la la la ....

Abbreviations

AE, anion exchange

AE1, Cl^-/HCO_3^- anion exchange protein 1

AE2, Cl^-/HCO_3^- anion exchange protein 2

AE3, Cl^-/HCO_3^- anion exchange protein 3

AE3c, cardiac isoform of AE3

AE3tr, truncated isoform of AE3

AP3, a polyclonal antibody directed against the common C-terminus of all AE3 variants

ATP, adenosine triphosphate

β, buffering capacity

β_{Total}, total intracellular buffer capacity

β_{CO2}, portion of buffering capacity mediated by CO_2/HCO_3^-

β_i, intrinsic buffer capacity, arising mainly from intracellular proteins

BCECF-AM, 2',7'–bis(2-carboxyethyl)–5(6)-carboxyfluorescein-acetoxymethyl ester

BIDS, 4-benzamido-4'-isothiocyanostilbene-2,2'-disulfonate

BLM , basolateral membrane

BTR-1, bicarbonate transporter related protein

C-terminus, carboxy-terminus

cAE3-1, a polyclonal antibody directed against the unique region of AE3c

CA, carbonic anhydrase

CaM, calmodulin

CCD, cortical collecting duct

CF, cystic fibrosis

CFTR, CF transmembrane conductance regulator

CHP, calcineurin B homolog protein

CLD, congenital chloride diarrhoea

cLPM, canalicular rat liver plasma membrane vesicles

DBDS, 4,4'-dibenzoylstilbene-2,2'-disulfonate

DIDS, 4,4´-di-isothiocyanostilbene-2,2´-disulphonate

DMEM, Dulbecco's modified Eagle medium

DNA, deoxyribonucleic acid

DNDS, 4,4'-dinitrostilbene-2,2'-disulfonate

DRA, downregulated in adenoma

DTSDT , human diastrophic dysplasia protein

EC, extracellular loop

ECL, enhanced chemiluminescence

EDTA, ethylenediaminetetraacetic acid

EIPA, ethylisopropyl amiloride

EMD 85131, 2-methyl-5-methylsulphonyl-1-(1-pyrrollyl)-benzoly-guanidine

EST, expressed sequence tag

GI, gastrointestinal

GPI, glycosylphosphatidylinositol

GST, Glutathione-S-Transferase

GST-AE1ct, Fusion of AE1 C-terminus to GST

GST-AE1EC3, fusion of third extracellular loop of AE1 to GST

GST-AE1EC4, fusion of fourth extracellular loop of AE1 to GST

GST-DRAct, fusion of DRA C-terminus to GST

H_2DIDS, 4,4'-diisothiocyanodihydrostilbene-2,2'-disulfonate

HEK, human embryonic kidney

HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

HOE 642, 4-isopropyl-3-methylsulphonylbenzoyl-guanadine methanesulphonate

IPTG, isopropylthiogalactoside

LB , Luria broth based media

MCT, monocarboxylate/H^+ co-transporter

N-terminus, amino-terminus

NAP-taurine, N-(4-azido-2-nitrophenyl)-2-aminoethanesulphonate

NBC, Na^+/HCO_3^- co-transporter

NCX, Na^+/Ca^{2+} exchanger

NDCBE, an NDAE protein cloned from human brain

NDAE, Na^+-dependent Cl^-/HCO_3^- anion exchange protein

NhaA, the bacterial Na^+/H^+ antiporter

NHE, Na^+/H^+ antiporter

PBS, phosphate buffered saline

PDS , Pendred's syndrome gene

pH_i, intracellular pH

pHo, extracellular pH

PKA, protein kinase A

PKC, protein kinase C

PMA, phorbol-12-myristate-13-acetate PMA

PMSF, phenylmethylsulfonyl fluoride

SAT-1, rat canalicular sulphate/bicarbonate transport protein

SDS, sodium dodecyl sulfate

SDS-PAGE, SDS-polyacrylamide gel electrophoresis

SPQ, 6-methoxy-N-(3-sulphopropyl)quinolinium

TLCK, N-p-tosyl-L-lysine chloromethyl ketone

TPCK, N-tosyl-L-phenylalanine

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Chapter 1

Introduction

Introduction

1.1 Intracelular pH

Intracellular pH (pH_i) is a major regulator of many cellular processes including metabolic pathways, Ca^{2+} homeostasis, cell contractility, cell excitability (Busa & Nuccitelli, 1984), gene expression (Isfort et al., 1993), and cell death (Reynolds, Li & Eastman, 1996). Therefore a tight control of pH_i is critical to maintain proper cell function.

The pH of a cell's surroundings and its sub-cellular compartments is determined by the concentration of H^+ ions in these locations. The distribution of H^+ ions across the plasma membrane of most cells is such that the pH_i is much higher than that predicted if H^+ were passively distributed across the membrane (Ellis & Thomas, 1976). For an average membrane potential of -60 mV and for an external pH of 7.4, a pH_i value of 6.4 would be expected if H^+ were in electrochemical equilibrium across the plasma membrane (Frelin et al., 1988). To maintain intracellular H^+ concentration, any passive influx of H^+, which is driven by the electrochemical gradient, must be counteracted by a H^+ efflux mediated by some kind of acid extruding membrane transport system. Extrusion of H^+ maintains pH_i at a value well above equilibrium, which is required for cytoplasmic reactions.

Changes in pH_i occur during metabolic and developmental transitions in many cells. For example, quiescent cells typically display an acidic cytoplasm (low pH_i), while cellular activation is often accompanied by alkalinisation (increased pH_i) (Busa & Nuccitelli, 1984; Roos & Boron, 1981). Such observations raise a number of important questions including:

(increased pH_i) (Busa & Nuccitelli, 1984; Roos & Boron, 1981). Such observations raise a number of important questions including:

- how is pH_i regulated?
- how are variations in pH_i achieved?
- how important is steady state pH_i for cell function?

The major pH_i regulatory systems in mammalian cells are the Na^+/H^+ exchange proteins (NHE) (Sardet et al., 1990; Sardet, Franchi & Pouyssegur, 1989), Cl^-/HCO_3^- anion exchange proteins (AE) (Kopito et al., 1989; Kopito & Lodish, 1985b; Lee, Gunn & Kopito, 1991) and Na^+/HCO_3^- co-transport proteins (NBC) (Burnham et al., 1997; Romero et al., 1997) (Figure 1.1 and Table 1.1). An intracellular buffering system also provides more immediate protection against changes in pH_i.

AE proteins facilitate the electroneutral exchange of chloride for bicarbonate across the plasma membrane. AE-mediated Cl^-/HCO_3^- exchange is bi-directional resulting in either the influx or efflux of HCO_3^- in exchange for Cl^- and provides a means for regulation of intracellular pH (pH_i), cell volume and intracellular Cl^- and HCO_3^- concentrations. The AE family is encoded by at least three genes, AE1, AE2 and AE3 (Alper, 1991), located on chromosomes 17, 7 and 2, respectively (Yannoukakos et al., 1994) (Figure 1.2). AE1 is the erythrocyte AE isoform and is also expressed in kidney, testis, heart, lung and liver (Brosius-III et al., 1989; Kudrycki, Newman & Shull, 1990; Kudrycki & Shull, 1989). AE2 is expressed in most tissues, at least at the mRNA level, (Alper et al., 1988; Demuth et al., 1986; Kudrycki et al., 1990) while AE3 expression is restricted to the brain,

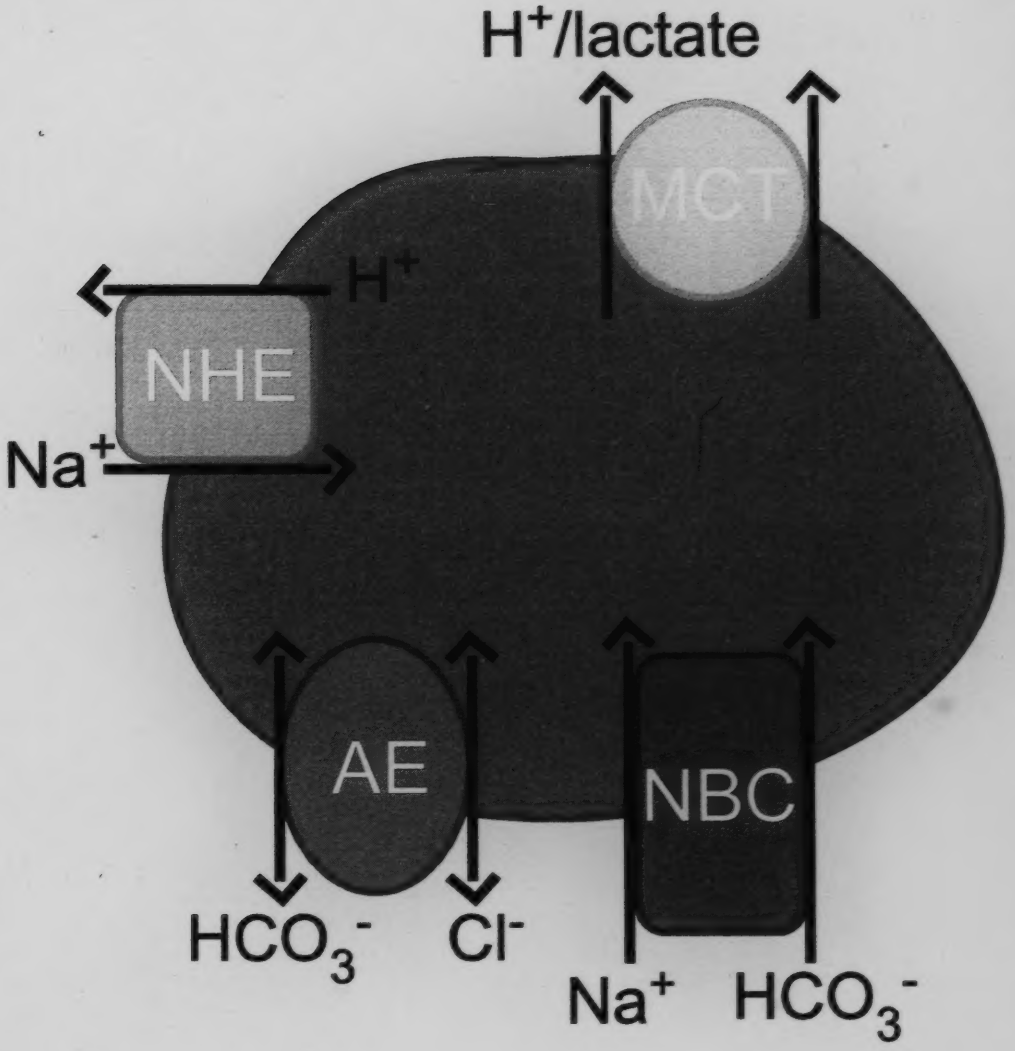
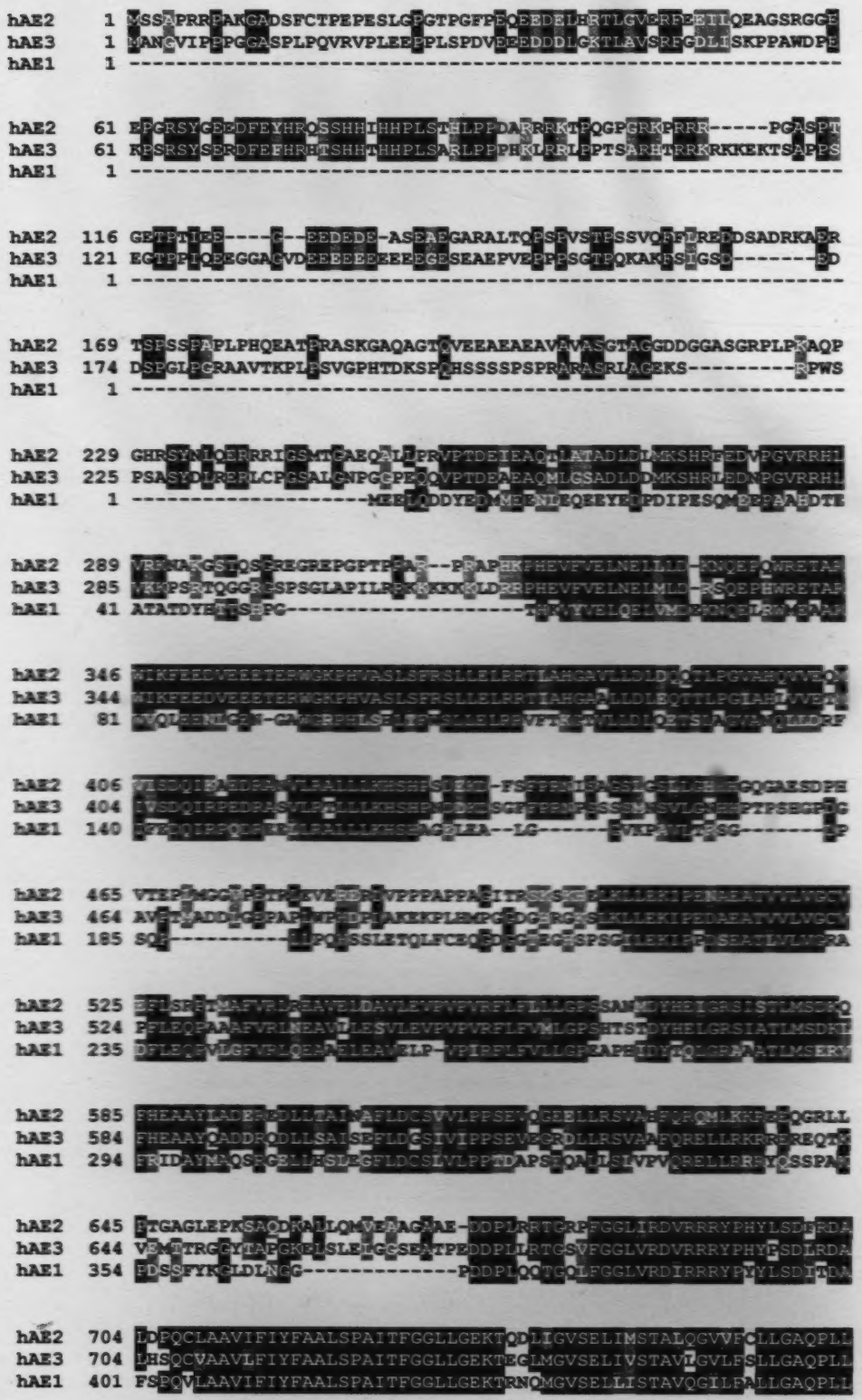


Figure 1.1 Main membrane transport proteins expressed in cardiomyocytes involved in pH_i regulation.



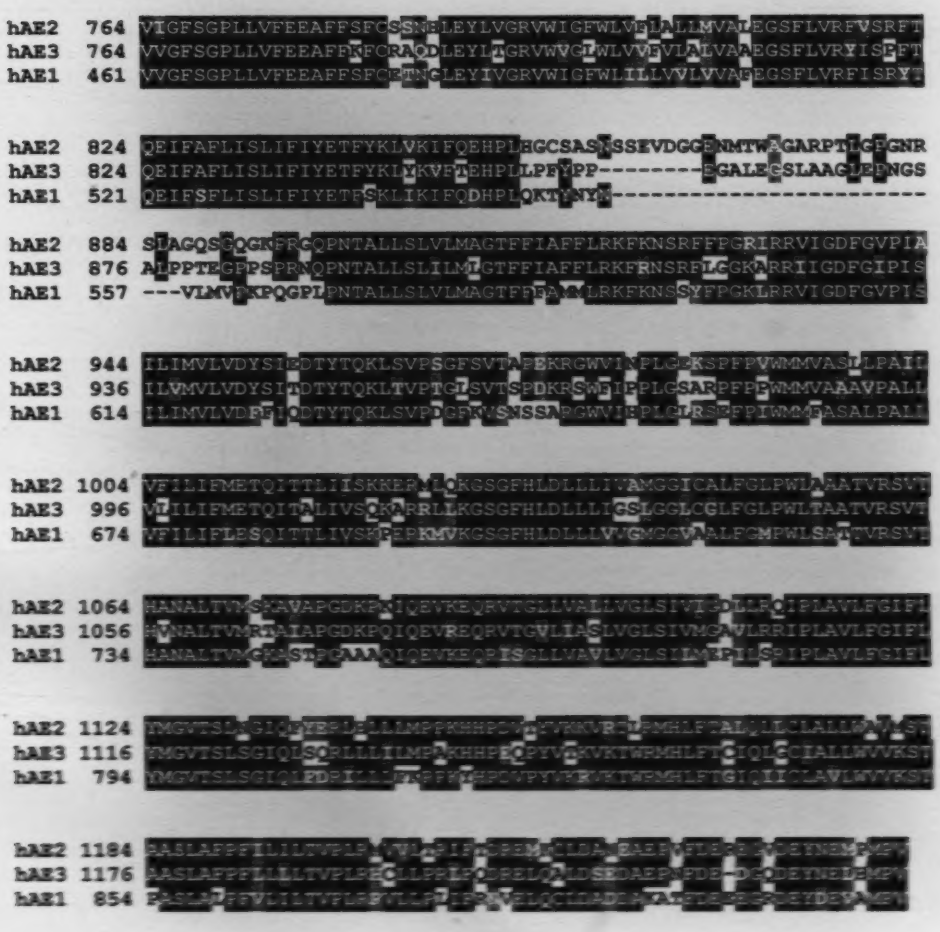


Figure 1.2 Amino acid sequence alignment of AE1, AE2, and AE3.

The first letter of each sequence name refers to the species: h (human). AE1, (Tanner, Martin & High, 1988); AE2, (Gehrig, Müller & Appelhans, 1992); AE3, (Yannoukakos et al., 1994).

heart and retina (Kobayashi et al., 1994; Kopito et al., 1989; Linn, Kudrycki & Shull, 1992). Coupled with the wide tissue distribution of AE proteins, their contribution to regulation of pH_i underscores their importance in the regulation of many cellular processes.

An extreme case of pH-related pathophysiology occurs in cardiomyocytes, the cells associated with contraction of the heart, during ischaemia followed by reperfusion. During an ischaemic episode, reduced blood flow leads to cellular acidosis from which cardiomyocytes must recover. Cellular acidosis affects the function of enzymatic processes in these cells, and has, in particular, a range of deleterious effects on the contractile apparatus (Orchard & Kentish, 1990). In addition, cellular acidosis is responsible, at least in part, for the reduced contractility found during circulatory failure and during reperfusion following ischaemia.

During an ischaemic attack, cells produce acid as a byproduct of metabolism, some of which is removed as lactate by the Monocarboxylate/H^+ co-transporter (MCT) (Poole & Halestrap, 1993). H^+ that are removed from the cell accumulate in the interstitial fluid as a consequence of reduced or absent perfusion. Thus, both intracellular and extracellular fluids become acidic. Upon reperfusion, extracellular acid is cleared leaving an outward-directed H^+ gradient, where intracellular [H^+] is greater than extracellular [H^+]. Figure 1.3 shows some of the pH_i recovery mechanisms employed by the heart, which include Na^+/H^+ exchange and Na^+/HCO_3^- co-transport. However, the transport mechanism employed by both of these proteins involves movement of Na^+ into the cell. Accumulation of intracellular Na^+ impairs the function of the Na^+/Ca^{2+}

exchange protein (NCX) (Cross et al., 1998), which leads, in turn to an accumulation of Ca^{2+} in the cytoplasm. Ca^{2+} overload manifests as cell necrosis, impaired contractility and arrhythmia (Donck, Borgers & Verdonck, 1993) (Figure 1.2).

NHE-1 may be a key factor in mediating a hypertrophic response, or cellular enlargening especially after a heart attack. NHE-1 is activated by mitogen activated protein (MAP) kinases in response to various paracrine and autocrine factors such as endothelin-1, angiotensin-II and α_1 adrenergic agonists (Moor & Fliegel, 1999; Wang et al., 1997), factors also known to induce hypertrophy. Inhibition of NHE-1 blocks hypertrophic responses to norepinephrine and stretch stimulation (Cingolani et al., 1998; Hori et al., 1990; Yamazaki et al., 1998). The NHE-1 specific inhibitor, 4-isopropyl-3-methylsulphonylbenzoyl-guanadine methanesulphonate (HOE 642), also known as cariporide, has beneficial effects in both early and late postinfarction-induced heart failure (Kusumoto, Haist & Karmazyn, 2001; Yoshida & Karmazyn, 2000). When administered orally, cariporide improves contractile dysfunction and abrogates hypertrophy following ischaemia (Yoshida & Karmazyn, 2000). Moreover, cariporide-mediated inhibition of NHE-1 also attenuates hypertrophy in a hypertension model (Chen et al., 2001).

Although the precise role of NHE-1 in hypertrophy remains to be determined the mechanism likely involves intracellular Na^+ accumulation following NHE activation. In addition, an increase in intracellular Na^+ activates protein kinase C (PKC), which in turn activates NHE-1 thus fostering a vicious cycle. As with inhibitors of NHE-1, inhibitors of PKC also reduce hypertrophic

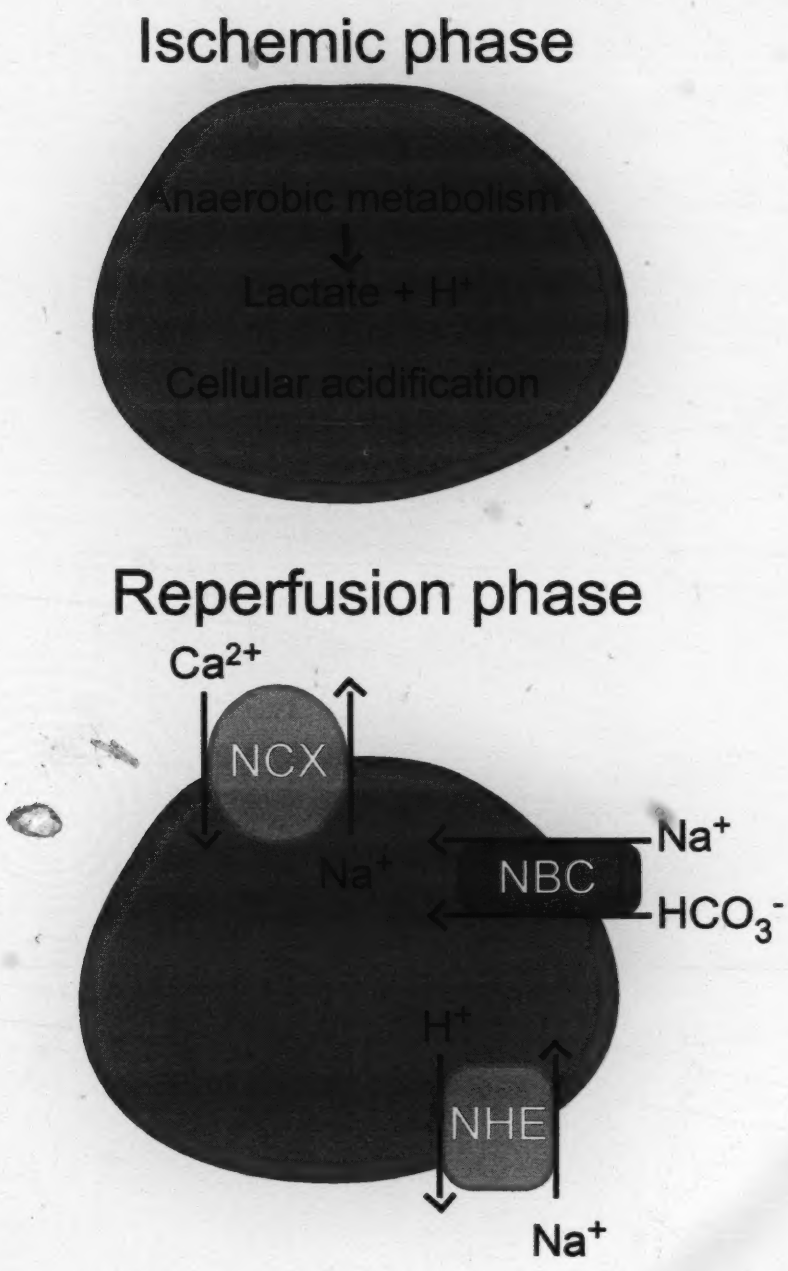


Figure 1.3 Role of acidification in ischaemia/reperfusion injury.

The top panel shows a cell during ischaemia and the bottom panel shows the same cell during reperfusion. The bottom panel indicates how during recovery of pH_i, NHE and NBC contribute to Na^+ overload and consequently impair the transport activity of NCX.

responses, reinforcing the link between PKC, NHE-1 and hypertrophy (Hayasaki-Kajiwara et al., 1999).

Inhibition of NHE-1 activity is currently being investigated as a clinical therapy. Cariporide, is now in clinical trials including the GUARDIAN trial involving almost 12,000 patients with acute coronary syndrome (Erhardt, 1999; Karmazyn, 2000; Theroux et al., 2000a; Theroux et al., 2000b). Since cardiac pH_i does not rise under hypertrophic conditions when NHE-1 is activated, it has been suggested that a parallel acidifying pathway is activated to counter NHE-1 activity during hypertrophy. Cardiac AE proteins have been proposed as the cell acidifiers (Alvarez, Fujinaga & Casey, 2001)

The AE family of proteins is growing in importance as key regulators of pH_i on the heart. Northern blots indicate that several isoforms of AE are expressed in the heart at levels AE3c >full-length AE3>>AE2>>AE1 (Alvarez et al., 2001). At the protein level, cardiomyocytes express two variants of both AE1 (Korichneva et al., 1995a; Pucéat et al., 1995; Richards et al., 1999) and AE3 (Linn et al., 1995). Hence, further understanding of the mechanisms used by these proteins to regulate pH_i may provide a means of therapeutic manipulation following an ischaemic episode

Importantly, unlike the mechanisms employed by NHE-1, regulation of pH_i by AE-mediated exchange of Cl^- for HCO_3^- does not contribute to intracellular Na^+ accumulation. As a result, AE-mediated recovery of pH_i following an ischaemic episode would not have the same deleterious effects on cardiomyocytes as the Na^+-coupled recovery mechanisms.

The incidence of heart attacks is rising dramatically, with death rates associated with heart attacks more than doubling in the past 10 years (Karmazyn, 2001). Therefore, an understanding of the contribution of AE proteins, to the regulation of pH_i in cardiomyocytes, is of fundamental importance and may provide a means for therapeutic manipulation of AE proteins especially during an ischaemic/reperfusion episode. The possibility that patients may soon have their NHE1 inhibited accentuates the need to understand the role of AE proteins in regulating pH_i in the heart even further. Identification of mechanisms that regulate anion exchanger activity is central to this thesis.

1.2 pH_i regulatory mechanisms

The major pH_i regulatory systems in mammalian cells are the Na^+/H^+ exchange proteins (Sardet et al., 1990; Sardet et al., 1989), Cl^-/HCO_3^- AE proteins (Kopito et al., 1989; Kopito & Lodish, 1985b; Lee et al., 1991) and Na^+/HCO_3^- co-transport proteins (Burnham et al., 1997; Romero et al., 1997) (Figure 1.3 and Table 1.1). In addition, an effective intracellular buffering system provides more immediate protection against changes in pH_i.

1.2.1 Intracellular buffer capacity

An intracellular buffering system provides protection against acute changes in pH_i. The ability to buffer pH_i changes in the short term is usually assessed experimentally by measuring buffer capacity (β), which is defined as the concentration of H^+ ions (moles/l) required to produce one pH unit change. The total intracellular buffer capacity, β_{total}, is classically divided into two parts, one mediated by HCO_3^-/CO_2 (β_{CO2}) and an intrinsic component, arising mainly

Table 1.1 Bicarbonate transport proteins.

A list of bicarbonate transport proteins identified to date with tissue distribution and transport mechanism.

Transport protein	Other name	Tissue distribution	Mechanism	Net charge movement	Reference
AE1	SLC4A1, Band 3	erythrocyte, kidney, heart	Cl^-/HCO_3^- exchange	0	(Kopito & Lodish, 1985a)
AE2	SLC4A2	widespread	Cl^-/HCO_3^- exchange	0	(Alper et al., 1988)
AE3	SLC4A3	brain, heart, retina, GIT, kidney	Cl^-/HCO_3^- exchange	0	(Kudrycki et al., 1990)
AE4	BTR	kidney	Cl^-/HCO_3^- exchange? Na^+/HCO_3^- co-transport?	?	(Tsuganezawa et al., 2001)
NBC1a	SLC4A4, kNBC	kidney, cornea	Na^+/HCO_3^- co-transport	-2 or -1	(Burnham et al., 1997; Romero et al., 1997)
NBC1b	SLC4A5, hhNBC, pNBC, Rb2NBC	heart, pancreas, kidney, cornea, prostate, colon, stomach, brain (glia), thyroid, spinal chord	Na^+/HCO_3^- co-transport	-2 or -1	(Abuladze et al., 1998; Choi et al., 1999)
NBC3 and splicing variants	SLC4A7, NBC2, NBCn1a,b,c,d	heart, kidney, skeletal muscle, pulmonary, artery, aorta, submandibular gland	Na^+/HCO_3^- co-transport	-2 or -1	(Ishibashi, Sasaki & Marumo, 1998; Pushkin et al., 1999)
NBC4		liver, spleen, epididymis, heart	Na^+/HCO_3^- co-transport		(Pushkin et al., 2000)
NDAE1	SLC4A8, NDCBE1	neurons, kidney, fibroblasts	Na^+-dependent Cl^-/HCO_3^- exchange	+1	(Grichtchenko et al., 2001; Romero et al., 2000)

SAT-1	SLC26A1	Liver, kidney, muscle, brain	SO_4^{2-}/HCO_3^- exchange		(Bissig et al., 1994; Palacin et al., 1990)
DRA	SLC26A3, CLD	colon, ileum	Cl^-/HCO_3^- exchange	0	(Melvin et al., 1999; Schweinfest et al., 1993)
Pendrin	SLC26A4, PDS	inner ear, thyroid, kidney	Cl^-/HCO_3^- exchange	0	(Scott & Karniski, 2000; Scott et al., 1999; Soleimani et al., 2001)
PAT-1	SLC26A6	kidney, heart, pancreas, liver intestine, skeletal muscle, placenta	Cl^-/HCO_3^- exchange	0	(Waldegger et al., 2001; Wang et al., 2002)
	SLC26A7	kidney	Cl^-, SO_4^{2-}, HCO_3^-?		(Lohi et al., 2002)
TAT-1	SLC26A8	spermatocytes	Cl^-, SO_4^{2-}, HCO_3^-?		(Lohi et al., 2002)
	SLC26A9	lung	Cl^-, SO_4^{2-}, HCO_3^-?		(Lohi et al., 2002)

from intracellular proteins (β_i) (Roos & Boron, 1981). The total buffering capacity of a cell (β_{total}) is determined as $\beta_{total} = \beta_i + \beta_{CO2}$, where $\beta_{CO2} = 2.3[HCO_3^-]$ (Roos & Boron, 1981).

Buffer capacity varies in different cell types. For example, the value of β_i in sheep cardiac purkinje fibres is about 20 mM/pH unit at a pH_i of 7.2 and increases linearly as pH_i becomes acidic (Vaughan-Jones, 1986). In HEK 293 cells however, β_i is negligible above pH_i 7.2 (Lee et al., 1991; Sterling & Casey, 1999).

1.2.2 Membrane transporters that regulate pH_i

A number of transport proteins are involved in regulating pH_i in a variety of cells and tissues. What follows is an overview of what is currently known about these proteins

1.2.2.1 Na^+/H^+ antiporters

Na^+/H^+ exchangers are universally employed by eukaryotic cells to regulate pH_i. As implied by the name, Na^+/H^+ exchangers transport H^+ ions outward across the plasma membrane in exchange for inward movement of Na^+ using energy provided by the inward-directed sodium gradient.

1.2.2.1.1 Cloning and tissue distribution

At least 7 NHE isoforms have been identified to date, with additional human genomic data indicating the existence of other putative NHE isoforms. Isoforms differ structurally and in their sensitivity to inhibition by pharmacological agents (Karmazyn & Moffat, 1993; Masayuki & Orlowski, 2001). The first NHE cDNA identified was isolated through a series of experiments involving complementation of NHE-deficient cell lines. This cDNA encodes the 110 kDa human housekeeping isoform, now referred to as NHE-1 (Sardet et al.,

1990; Sardet et al., 1989). Cloning of NHE-1 provided the means to identify other members of the NHE gene family by low stringency hybridisation of cDNA libraries using NHE-1 cDNA as a probe. NHE-1 has a message size of 5.0-5.4 kb and appears to be ubiquitously expressed. NHE-1 resides in the basolateral membrane (BLM) of many epithelial cells as well as in the plasma membrane of non-polarised cells and is the predominant isoform found in the mammalian heart (Fliegel et al., 1993).

In general, the NHE isoforms have a wide tissue distribution. NHE-2 is expressed predominantly in the apical membrane of renal epithelia and in gastrointestinal epithelia including small intestine, colon and stomach where it participates in trans-epithelial NaCl transport. NHE-2 is also expressed in non-epithelial tissues, such as trachea and smooth muscle (Counillon & Pouyssegur, 1995). NHE-3 is expressed only in epithelia including that of kidney, intestine and stomach while NHE-4 occurs at high levels in the stomach and at lower levels in the small and large intestine (Counillon & Pouyssegur, 1995). Unlike the other isoforms, NHE-5 resides in a select number of non-epithelial tissues (brain > spleen >> testis > skeletal muscle) (Klanke et al., 1995) and may represent the amiloride insensitive NHE variant reported in hippocampal neurons (Raley-Susman et al., 1991). NHE-6 is expressed in several human tissues examined, with the highest levels found in brain and skeletal muscle (Orlowski & Grinstein, 1997). NHE-7 is ubiquitously expressed and localises predominantly to the *trans*-Golgi network (Masayuki & Orlowski, 2001).

1.2.2.1.2 NHE structural features

Hydropathy analysis and sequence comparison of the NHE gene family reveal several structural features of the Na^+/H^+ exchangers (Fliegel & Frohlich, 1993; Sardet et al., 1989). All NHE proteins possess two separate functional domains: an N-terminal hydrophobic domain that spans the membrane 10-12 times (Counillon & Pouyssegur, 1995; Fliegel & Frohlich, 1993; Orlowski & Grinstein, 1997; Sardet et al., 1989) and is necessary for the ion transport process, and a hydrophilic cytoplasmic domain (Counillon & Pouyssegur, 1995; Fliegel & Frohlich, 1993; Orlowski & Grinstein, 1997; Sardet et al., 1989) that is crucial for determining the set point value of the H^+ sensor system of the exchangers and is required for the hormonal regulation of the proteins (Counillon & Pouyssegur, 1995; Takaichi et al., 1993).

1.2.2.1.3 NHE stoichiometry

In purified renal cortex microvillus membrane vesicles, Na^+ influx is stimulated by outwardly directed H^+ gradients and H^+ efflux is stimulated by an inwardly directed Na^+ influx. Neither process is altered by differences in membrane potential, indicating an electroneutral exchange of Na^+ for H^+ (Kinsella & Aronson, 1980). Imposing an outwardly directed Na^+ gradient, thermodynamically coupled to an outwardly directed H^+ gradient of equal magnitude, abolishes net Na^+ uptake, which suggests a stoichiometry of Na^+ for H^+ of 1:1 (Kinsella & Aronson, 1982). Consistent with this suggested stoichiometry, vesicle Na^+ uptake follows simple hyperbolic kinetics with respect to external Na^+ concentration, indicating a single external binding site for Na^+ (Aronson, 1983). Taken together these results suggest that in mammals, Na^+/H^+

antiporters function as a one for one electroneutral exchanger of sodium ions for protons.

In bacteria, electrogenic Na^+/H^+ exchange is mediated by NhaA, the bacterial Na^+/H^+ antiporter (Karpel et al., 1988). Experimentally, NhaA electrogenicity was determined by reconstituting the NhaA protein into sodium-loaded proteoliposomes and monitoring Na^+ efflux and H^+ influx. The stoichiometry was then calculated from the ratio of the ion fluxes. Another experimental approach was based on measuring the membrane potential generated by NhaA at various sodium gradients, assuming a complete coupling and thermodynamic equilibrium between the membrane potential and the ion gradients. The results from both methods agree with a stoichiometry of two H^+ exchanged for each Na^+ through a process that is independent of pH between pH 7.2 and 8.0 (Taglicht, Padan & Schuldiner, 1993).

1.2.2.1.4 Cation specificity of NHE

NHE is highly selective for Na^+. However, the presence of other extracellular cations, including H^+, Li^+ and NH_4^+ inhibits H^+_i-activated $^{22}Na^+$ influx in a competitive manner and these cations can replace Na^+ in Na^+/H^+ exchange (Aronson, 1985). The presence of extracellular H^+ or Li^+ inhibits $^{22}Na^+$ influx in cells expressing either NHE-1 or -3 by interacting at a single binding site. However in contrast to extracellular H^+ and Li^+, extracellular K^+ inhibits NHE-1 but not NHE-3 activity and cannot substitute for Na^+ as a substrate for Na^+/H^+ exchange (Orlowski, 1993).

1.2.2.1.5 Inhibition of NHE

NHE-1 is inhibited on the extracellular side by the diuretic drug amiloride and it's derivatives, which compete with sodium at the transport site (Takaichi et al., 1993). Although also inhibited by amiloride derivatives, NHE-3 is less sensitive than NHE-1 with a range of potency varying between 62- and 608-fold depending on the analogue used. The susceptibility varies between these NHE isoforms, however the order of sensitivity to amiloride derivatives is similar for both isoforms:

NHE-1 EIPA ≥ DMA >> amiloride >> benzamil

NHE-3 EIPA > DMA > amiloride ~ benzamil (Orlowski, 1993)

In addition to amiloride analogues, other pharmacological agents are also known to inhibit the exchanger family. These agents include cimetidine, a histimine H_2-receptor antagonist, clonidine, an α_2-adrenergic receptor agonist, harmaline, a hallucinogenic drug known to inhibit amine oxidase and antagonise some Na^+-dependant transport systems (Kulanthaivel et al., 1990), loperamide, an opiate receptor agonist (Balkovetz et al., 1987) and various derivatives of guanidinium (Frelin et al., 1986). While these compounds are chemically unrelated to amiloride they generally possess either an imidazoline or guanidinium moiety and hence bear some structural resemblance to amiloride. A comparison of a number of these inhibitors indicates that, as with amiloride, NHE-1 is more susceptible to inhibition than is NHE-3 with a degree of potency ranging from 3 to 238-fold. However, unlike amiloride analogues the order of inhibitory potency of these compounds varies between isoforms:

NHE-1 cimetidine > clonidine ≥ harmaline

NHE-3 clonidine ≥ harmaline > cimetidine (Orlowski, 1993)

Over the last decade a new class of NHE inhibitors that are not directly related to the amiloride structure has emerged. The most studied of these is HOE 642 or cariporide. Cariporide is an isoform-specific inhibitor of NHE-1, the most predominant NHE isoform in the heart (Scholz et al., 1995). Inhibition of NHE-1 during/after an ischaemic episode results in improved cardiac function compared with controls (Myers & Karmazyn, 1996). Following many promising studies it is now accepted that inhibition of NHE-1 by cariporide attenuates cardiac hypertrophy (Karmazyn, 2001) and confers cardiac protection during ischaemia and reperfusion (Gumina, Terzic & Gross, 2001). The development of similar compounds such as EMD 85131 (2-methyl-5-methylsulphonyl-1-(1-pyrrollyl)-benzoly-guanidine) that have similar cardioprotective effects and the entry of NHE inhibitors into clinical trials provides an immediate need to understand other pH regulating mechanisms in the heart in the absence of functional NHE-1 (Erhardt, 1999; Karmazyn, 2000; Theroux et al., 2000a; Theroux et al., 2000b).

1.2.2.1.6 pH sensitivity and regulation of NHE

Na^+/H^+ exchange is subject to regulation on two levels: (i) through modification of exchanger turnover rate and (ii) through mechanisms that modulate the number of exchangers available for transport. On the first level, cytosolic [H^+] modulates Na^+/H^+ exchanger turnover rate in two ways. H^+ are both substrates and allosteric regulators, thus giving rise to the steep activation of exchange at a lowered intracellular pH. This sensitivity allows the protein to adjust its turnover rate to changes in intracellular pH (Aronson, Nee & Suhm, 1982). As a consequence of the steep activation curve at lowered pH_i, it is

reasonable to assume that the Na^+/H^+ exchanger is only marginally active at resting pH.

In addition to the effects of $[H^+]$, NHE turnover rate is also modified by hormones and mitogens, which modulate NHE activity by activation of kinases that phosphorylate NHE. The cytosolic domains of members of the NHE family have consensus sites for phosphorylation by PKC and/or PKA as well as multiple sites that are suitable for CaM kinases and other Ser/Thr kinases (Fliegel & Frohlich, 1993). The latter include the mitogen-activated protein kinases, which have been implicated in the activation of NHE by causing a shift of the pH activation curve to more alkaline pH (Aharonovitz & Granot, 1996; Bianchini, L'Allemain & Pouyssegur, 1997; Moor & Fliegel, 1999).

The cytoplasmic C-terminal tail of NHE-1 contains two regions capable of binding calmodulin with high (CaM-A K_d ~20 nM) and low (CaM-B Kd~350 nM) affinity. Deletion of the high affinity site renders the protein constitutively active, as if the cytosolic $[Ca^{2+}]$ were continuously elevated. It has therefore been suggested that at basal $[Ca^{2+}]$, the unoccupied CaM-A site exerts an auto-inhibitory affect on the antiporter that is relieved upon ligand binding (Bertrand et al., 1994). Of the NHE family, only NHE-1 is regulated by CaM. However, insertion of the CaM-binding site of NHE-1 into the C-terminal tail of NHE-3, confers $[Ca^{2+}]$ sensitivity to NHE-3. With this modification, the transmembrane region of NHE-3 then responds to conformational changes of the C-terminal tail induced by CaM binding in the same manner as NHE-1 (Wakabayashi et al., 1995).

A second calcium binding protein that interacts with NHE-1 is the calcineurin B homolog protein (CHP) which exerts an inhibitory effect on the antiporter (Lin & Barber, 1996). CHP appears to be constitutively phosphorylated and its dephosphorylation correlates with stimulation of NHE-1 activity. This prompted the suggestion that the phosphorylated CHP protein is normally associated with the antiporter, exerting a suppressive effect while dephosphorylation of CHP leads to dissociation from NHE and thus activation of Na^+/H^+ exchange activity (Lin & Barber, 1996).

The second level of regulation of Na^+/H^+ exchanger activity depends on modulating the number of exchangers available for transport. Investigations of the transcriptional regulation of NHE-1 indicate that a proximal region of the NHE-1 gene containing an AP-2 binding site (Yang et al., 1996), a novel poly (ddA:dAT) region of the gene (Yang et al., 1996) and the transcription factor COUP (Fernandez-Rachubinski & Fliegel, 2001) are important in regulating expression of the antiporter.

1.2.2.2 Monocarboxylate/H^+ co-transporters

Transport of the monocarboxylic acid, lactate across the plasma membrane is important to most mammalian cells. Under anoxic conditions, or in tissues with few or no mitochondria, such as white muscle and red blood cells, lactate is produced as the end product of glycolysis and must be expelled from the cell. By contrast, under normal aerobic conditions in cells with mitochondria, little metabolic lactate is produced and must be imported. In these cells, lactate is brought in and then either oxidised as a respiratory fuel in

erythrocytes and cardiac and skeletal muscle, or converted into glucose by the liver and kidney.

Monocarboxylic acids such as acetate and butyrate can move across the plasma membrane by free diffusion of the undissociated acid (Walter & Gutknecht., 1984). The same is true for pyruvate at high concentration (Bakker & Dam., 1974). However at the concentrations occurring physiologically, the rates of diffusion are very slow since monocarboxylic acids are almost entirely in their dissociated anionic forms at physiological pH, hence retarding their movement across the membrane. A specific carrier-mediated mechanism for lactate co-transport is thus required. Such a carrier-mediated mechanism was demonstrated in 1974 with inhibitor studies (Halestrap, Brand & Denton, 1974; Halestrap & Denton, 1974), which indicates that a monocarboxylate is co-transported along with a H^+ across the plasma membrane.

It is now recognised that lactate and pyruvate enter the red blood cell by three routes: (i) diffusion of the free acid, (ii) transport on a specific monocarboxylate/H^+ co-transporter (MCT), or (iii) in exchange for another anion such as Cl^- or HCO_3^- on the anion exchanger protein (AE) when present at high concentrations. These pathways are distinguishable by the use of inhibitors.

1.2.2.2.1 Inhibition and cloning

Studies performed with cardiomyocytes have indicated a difference in inhibition of MCT activity with both cinnamates and sulphonates (Poole & Halestrap, 1993). From these results it was inferred that there are two different isoforms of monocarboxylate transporter in cardiomyocytes. At the molecular level two different isoforms of MCT have been found in the heart. The first

isoform, MCT-1, was cloned serendipitously while looking for a mevalonate transporter. MCT-1 was subsequently used as a probe for low stringency screening of a hamster liver cDNA library, yielding a cDNA encoding the second isoform of the monocarboxylate transporter, MCT-2 (Garcia et al., 1995).

The predicted amino acid sequence of MCT-2 is 60% identical to MCT-1 and hydropathy plots for the two proteins are practically superimposable. Northern blots indicated that the heart expresses both MCT-1 and MCT-2, which suggests that each has a different function in cardiomyocytes. MCT-2 was also found in parietal cells and in the renal medulla (Garcia et al., 1995), both of which are acidic environments. This implies that perhaps MCT-2 is active at an acid pH. In this case MCT-1 regulates small changes in pH_i in cardiac cells and MCT-2 could then be induced to aid in recovery from a significant acid load.

1.2.2.3 Na^+/HCO_3^- co-transporters

Na^+/HCO_3^- co-transporters mediate both electroneutral and electrogenic movement of Na^+ and HCO_3^- together across the plasma membrane to contribute to the regulation of pH_i. Although much information has been gathered on the molecular properties of NHE and AE isoforms, the molecular properties of NBC have not been thoroughly characterised to date. Recent molecular cloning experiments have identified the presence of several NBC isoforms (Table 1.1), which display distinct tissue distribution and differential regulation under pathophysiological conditions.

1.2.2.3.1 Cloning and tissue distribution

Three human NBC isoforms have been identified to date, two of which have 5'-end variants. NBC1a, the first mammalian sequence to be reported, was

cloned from human kidney using a molecular probe that had been identified by searching for sequences similar to the anion exchanger in the 'expressed sequence tag' database section of GenBank (Burnham et al., 1997; Romero et al., 1997). Human kidney NBC1a also gave a strong hybridisation signal on a human pancreas northern blot, suggesting it is also found there. Subsequently, a second NBC1 cDNA, NBC1b, was cloned from human pancreas.

NBC1b differs from NBC1a by a unique 5' end including 85 amino acids in an open reading frame that replaces the first 41 amino acids of the human kidney sequence (Abuladze et al., 1998). When the 5' ends of the two variants were used to probe multiple-tissue Northern blots, the kidney variant hydridised only with kidney mRNA, whereas the pancreas variant gave strong signals with mRNA from prostate, colon, stomach, thyroid, brain, spinal chord, pancreas and heart. Because the two amino acid sequences differ by less than 1% overall, the two variants are likely to be encoded by the same gene.

NBC3 was cloned from human skeletal muscle (Pushkin et al., 1999). Northern blot analysis in a variety of tissues gave multiple transcript sizes for NBC3; a 9 kb transcript expressed in brain spinal chord and adrenal gland (Amlal, Burnham & Soleimani, 1999), a ~7.8 kb transcript expressed in skeletal muscle and heart (Pushkin et al., 1999), an intermediate 4.4 kb transcript highly expressed in brain and spinal chord and moderately in thyroid, trachea and kidney (Amlal et al., 1999). Overall NBC3 has an amino acid sequence that shares 39 and 46% identity with NBC1a and NBC1b respectively.

NBC4 was cloned from human heart and maps to chromosome 2p13. The ~6kb transcript is moderately expressed in heart, with the highest expression

observed in liver, testes and spleen (Pushkin et al., 2000). At the amino acid level, NBC4 is 56% identical to NBC1 and 40% identical to NBC3 (Sassani et al., 2002). Unlike other members of the NBC family, NBC4 has a unique glycine rich region (amino acids 438-485). In addition, NBC4 lacks the lysine rich C-terminus of NBC1 (Pushkin et al., 2000).

1.2.2.3.2 Stoichiometry of NBC proteins

1.2.2.3.2.1 Kidney

On the basis of the electrochemical gradients of Na^+ and HCO_3^- across the BLM of the kidney proximal tubule, there should be a net flux of HCO_3^- from blood to the proximal tubule cell if NBC carries one Na^+ for one HCO_3^-. However, in all studies to date, NBC transport in the kidney has been found to be electrogenic and associated with a net flux of negative charge (Aronson, Soleimani & Grassl, 1991; Boron & Boulpaep, 1989; Soleimani & Burnham, 2001). The greater the HCO_3^- per Na^+ stoichiometry, and hence the greater the net negative charge movement per transport event, the more effectively the negative potential of the inner membrane of the cell can drive the net exit of HCO_3^-. Some studies indicate that the membrane potential alone would be sufficient to drive net HCO_3^- efflux across the BLM of the proximal tubule under physiological conditions if the stoichiometry of co-transport was 2 HCO_3^- : Na^+ (Yoshitomi, Burckhardt & Fromter, 1985). However, two separate studies, one in perfused rat proximal tubule and the other in rabbit BLM vesicles, showed that renal NBC has an apparent stoichiometry of 3 HCO_3^- for each Na^+ (Soleimani, Grassl & Aronson, 1987; Yoshitomi et al., 1985). With such a stoichiometry, the negative inside membrane potential, normally around –60 mV, is sufficient to drive HCO_3^-

efflux against the inward directed concentration gradients of HCO_3^- and Na^+ in the proximal tubule.

Although the results of these studies indicate a stoichiometry of 3 HCO_3^- for each Na^+ ion, they are equally consistent with a net transfer of three base equivalents and one Na^+. The nature of the base species transported by NBC was studied using the ^{22}Na influx method in BLM vesicles isolated from rabbit renal cortex (Soleimani & Aronson, 1989b). The results showed that $^{22}Na^+$ influx was stimulated when [CO_3^{2-}] was increased at constant [HCO_3^-], indicating a transport site for CO_3^{2-}. The results further showed that the binding of HCO_3^- to a distinct site in NBC was necessary for binding of CO_3^{2-} to its corresponding site (Soleimani & Aronson, 1989b). These studies led to the conclusion that efflux of HCO_3^- across the BLM of proximal tubule occurs via co-transport of 1 CO_3^{2-} : 1 HCO_3^- : 1 Na^+ at distinct sites (Soleimani & Aronson, 1989b), consistent with a stoichiometry of three base equivalents per Na^+ (Soleimani et al., 1987). Phosphorylation of Ser982 in the C-terminus of NBC1a by PKA, shifted the HCO_3^- : Na^+ stoichiometry of NBC1a from 3 : 1 to 2 : 1. This change in stoichiometry is predicted to cause a shift in the direction of Na^+ and HCO_3^- in the renal tubule BLM from efflux to influx (Gross et al., 2001).

1.2.2.3.2.2 Nonrenal tissues

A major difference in the role of NBC in renal verses nonrenal tissues is the direction of transport. In the proximal tubule, NBC mediates the efflux of HCO_3^- across the BLM to the blood, functioning as an acid loader (Aronson et al., 1991; Boron & Boulpaep, 1989). However, in other tissues such as the heart and liver, NBC mediates the influx of HCO_3^- from blood into the cell leading to cell

alkalinisation (Fitz, Persico & Scharschmidt, 1989; Lagadic-Gossmann, Buckler & Vaughan-Jones, 1992). Furthermore, unlike kidney NBC1 which has a stoichiometry of 3 base equivalents per Na^+, the brain NBC has a stoichiometry of two base equivalents per Na^+ (Aronson et al., 1991; Boron & Boulpaep, 1989). Electrophysiological measurements of cardiac NBC activity suggested a coupling stoichiometry of 2 HCO_3^+ : 1 Na^+ (Camilion de Hurtado et al., 1996). At the molecular level, two NBCs have been identified in the heart, NBC1 and NBC3. NBC1 and NBC3 have HCO_3^+ : Na^+ coupling ratios of 2/3:1 and 1:1, respectively (Choi et al., 1999; Pushkin et al., 1999).

1.2.2.3.3 Cation and anion specificity of NBC

The cation specificity of NBC has been studied in cortical BLM vesicles. Other than Na^+, NBC showed significant affinity only for Li^+, although this affinity was one fifth of that for Na^+ (Soleimani & Aronson, 1989b; Soleimani et al., 1991a).

The anion specificity of NBC has been studied in proximal tubule BLM. In the presence of HCO_3^-, ^{22}Na influx was significantly stimulated by sulphite (Soleimani & Aronson, 1989b). Increasing [CO_3^{2-}] at constant [HCO_3^-] reduced the stimulation of ^{22}Na influx by SO_3^{2-}, suggesting competition between SO_3^{2-} and CO_3^{2-}. NBC showed no affinity for sulphate or phosphate. Moreover, imposing an inward pH gradient in the absence of HCO_3^- resulted in no significant ^{22}Na uptake in rabbit renal BLM vesicles indicating that kidney NBC does not accept OH^- (Soleimani & Aronson, 1989a). By contrast, NBC in the colon has been suggested to accept OH^- in place of HCO_3^- (Rajendran, Oesterlin & Binder, 1991).

1.2.2.3.4 Inhibition of NBC proteins

The disulphonic stilbene derivatives DIDS and DNDS both inhibit NBC1 (Soleimani, Hattabaugh & Bizal, 1994). In addition, Harmaline inhibits the co-transporter by competitive interaction with Na^+ (Soleimani & Aronson, 1989b) and Furosemide and bumetanide also inhibit the co-transporter (Grassl, Holohan & Ross, 1987). NBC3 on the other hand is not sensitive to DIDS, but is inhibited by amiloride and its derivatives (Pushkin et al., 2000).

Several studies have also demonstrated that the carbonic anhydrase inhibitor, acetazolamide, reduces the rate of H^+ secretion and HCO_3^- reabsorption in the proximal tubule by >80%. These studies indicate that acetazolamide can inhibit NBC by decreasing the substrate availability at the inner surface of the BLM (Lucci et al., 1980; Lucci, Warnock & Rector, 1979; Soleimani & Burnham, 2001).

1.2.2.3.5 pH sensitivity and regulation of NBC

Regulation of NBC activity by pH_i has been studied in BLM vesicles by examining the effect of internal pH on HCO_3^--dependant $^{22}Na^+$ efflux (Soleimani et al., 1991b). Net influx of Na^+ was significantly higher at pH_i 7.2 than at pH_i 8.0, despite the higher $[HCO_3^-]$ and $[CO_3^{2-}]$ at this pH_i. Increasing the concentration of HCO_3^- and CO_3^{2-} at steady state pH_i had no effect on NBC transport activity, indicating that it was the change in pH_i and not substrate concentration that inhibited the co-transporter at alkaline pH_i. These results are consistent with the presence of a modifier site, similar to the H^+ modifier of NHE that inhibits NBC at alkaline pH_i. The combination of modifier sites on the luminal NHE-3 and the basolateral NBC should help maintain intracellular pH despite changes in extracellular pH (Soleimani et al., 1991b).

1.2.2.4 Na^+-dependent Cl^-/HCO_3^- anion exchangers

Na^+-dependent Cl^-/HCO_3^- anion exchange (NDAE) activity has been characterised in neurons (Russell & Boron, 1976; Schwiening & Boron, 1994; Thomas, 1977), kidney (Ganz et al., 1989; Guggino et al., 1983), heart (Liu, Piwnica-Worms & Lieberman, 1990) and fibroblasts (Kaplan & Boron, 1994). Although a functional activity of NDAE had been shown for many years, the molecular identity of the protein responsible was only recently discovered when a Na^+-dependent Cl^-/HCO_3^- anion exchange protein (NDAE1) was cloned from *Drosophila* (Romero et al., 2000). The *Drosophila* cDNA encodes a 1030 amino acid protein with both sequence homology and predicted topology similar to the AE and NBC proteins. NDAE1 mRNA is expressed throughout *Drosophila* and is prominent in the CNS. When expressed in *X. laevis* oocytes, *Drosophila* NDAE1 displays all the physiologic properties of a Na^+-dependent Cl^-/HCO_3^- exchanger: Cl^- transport, Na^+ transport, Na^+/HCO_3^- co-transport and sensitivity to DIDS and the data indicate that NDAE1 exchanges Na^+ and HCO_3^- (or an anion) for Cl^- and H^+. The authors postulate that NDAE1 may aid in maintaining the hind-gut pH of *Drosophila*.

The NDAE protein cloned from human brain is called NDCBE (Grichtchenko et al., 2001). Northern blots of multiple human tissues indicate that NDCBE is expressed in brain, testes and kidney and expression of NDCBE in *X. laevis* oocytes results in NDAE transport activity (Grichtchenko et al., 2001).

Transport activity of NDAE is regulated by many factors and has been shown to increase during renal development (Ganz & Saksa, 1998). In addition, a number of factors stimulate NDAE transport activity in mesangial cells including angiotensin II, serotonin, vasopressin, epidermal growth factor and

platelet-derived growth factor, (Ganz & Boron, 1994; Ganz et al., 1989). Transformation of NIH-3T3 fibroblasts with c-Ha-ras not only increased NDAE-exchange activity, but also shifted the activation to more alkaline pH values, effectively removing pH_i as the transporter control mechanism (Kaplan & Boron, 1994).

1.2.2.5 Cl^-/HCO_3^- anion exchangers

The erythrocyte Cl^-/HCO_3^- exchanger was first cloned in 1985 (Kopito & Lodish, 1985a). Due to its abundance in erythrocytes (50% of integral membrane protein), AE1 is one of the most extensively studied membrane transport proteins. Cl^-/HCO_3^- anion exchange proteins transport Cl^- and HCO_3^- ions across the plasma membrane in opposite directions, thus contributing to the regulation of pH_i, $[Cl^-]$ and cell volume.

AE1 has several functions specifically within the erythrocyte. The main function of erythrocyte AE1 is to increase the CO_2 carrying capacity of blood. In peripheral tissues, metabolically produced CO_2 can diffuse across membranes into the blood plasma. Under normal physiological conditions, the plasma becomes rapidly saturated as a consequence of the poor solubility of CO_2. To increase the CO_2-carrying capacity of the blood, CO_2 is hydrated to the more soluble HCO_3^- through a process mediated by the enzyme carbonic anhydrase, which is localised in erythrocytes. Although HCO_3^- is more soluble than CO_2, it is membrane-impermeant and would accumulate to a high concentration in the erythrocyte in the absence of AE1 activity. The erythrocyte AE isoform, AE1, mediates the exchange of internal HCO_3^- for external Cl^-, maintaining electroneutrality and osmotic balance. Thus CO_2 can be effectively moved

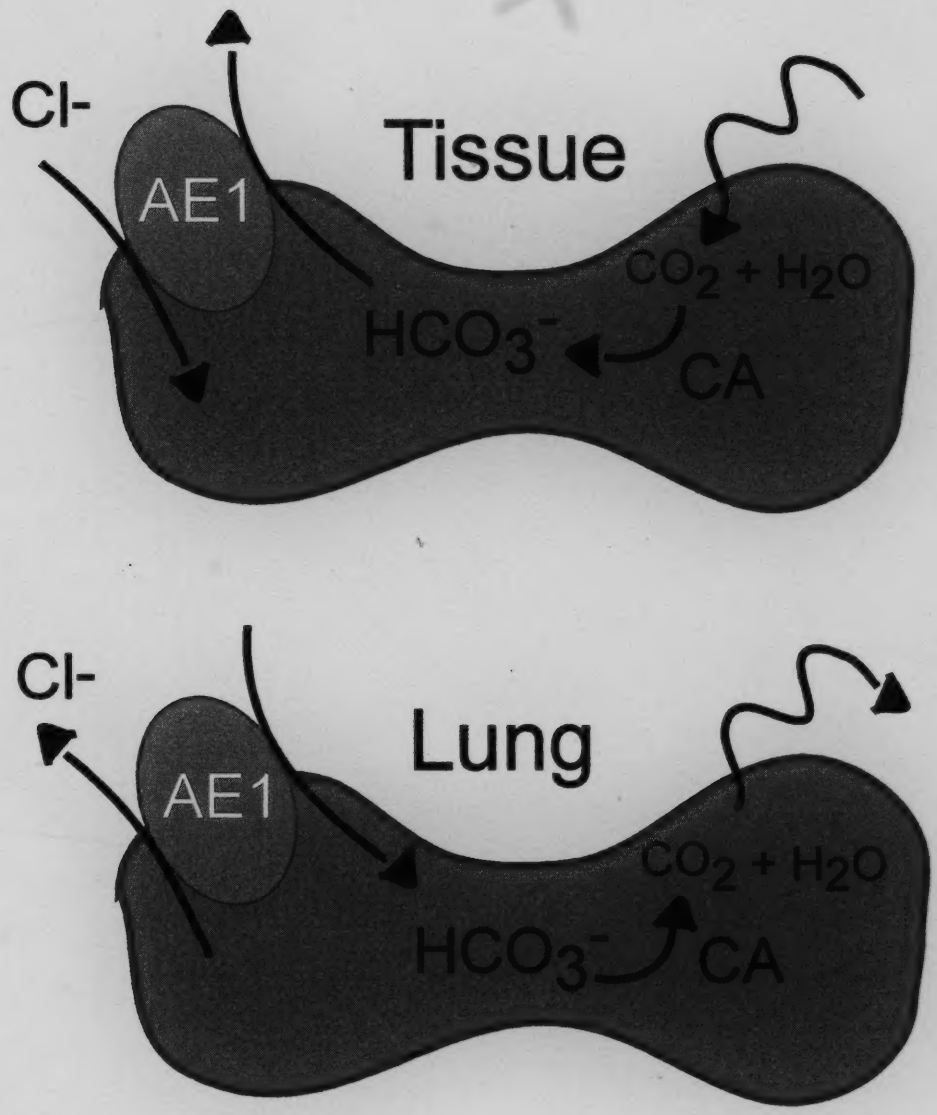


Figure 1.4 Clearance of CO_2 from the body.
The upper panel shows an erythrocyte in peripheral tissues and the lower panel shows an erythrocyte in the lungs.

around the body in the blood plasma as HCO_3^-. To remove CO_2 from the body, the process is reversed in the lungs. Since the pCO_2 is low in the lungs, mass action drives the conversion of HCO_3^- to CO_2. HCO_3^- therefore enters the erythrocyte in exchange for intracellular Cl^-. In the erythrocyte, carbonic anhydrase dehydrates HCO_3^- to CO_2, which can thus diffuse across the erythrocyte plasma membrane to the lung for expiration (Figure 1.4).

After anion exchange, the second important role of AE1 is to anchor the erythrocyte spectrin/ankyrin skeleton to the plasma membrane. The interaction between the N-terminal cytoplasmic domain of AE1 and the erythrocyte cytoskeleton is mediated by ankyrin and maintains the erythrocyte's characteristic biconcave disc shape.

1.2.2.5.1 Cloning and tissue distribution

The AEs are ubiquitously distributed in vertebrate tissues. The family is encoded by three genes, AE1, AE2 and AE3 located on chromosomes, 17, 7 and 2, respectively (Yannoukakos et al., 1994). All three AE genes give rise to multiple mRNA transcripts that vary in length and in their 5' non-coding and coding sequences (Figure 1.5) (Brosius-III et al., 1989; Demuth et al., 1986; Kopito & Lodish, 1985a; Kudrycki et al., 1990; Kudrycki & Shull, 1989; Linn et al., 1992; Wang, Schultheis & Shull, 1996). The three isoforms share 65% amino acid sequence homology in the transmembrane region, diverging to a greater extent in the N-terminal cytoplasmic region (Alper, 1991) (Figure 1.2).

1.2.2.5.1.1 AE1

Although AE1 was first cloned from mouse (Kopito & Lodish, 1985a), AE1 cDNAs have also been isolated from chicken (Cox & Lazarides, 1988), rat

(Kudrycki & Shull, 1989), trout (Hubner et al., 1992) and human (Lux et al., 1989; Tanner et al., 1988). Northern blots have shown AE1 transcripts in kidney and testis with low levels also detected in heart, lung and liver (Brosius-III et al., 1989; Kudrycki et al., 1990; Kudrycki & Shull, 1989). Two variants of AE1, eAE1 and kAE1, have been cloned and are the major protein isoforms present in erythrocyte and kidney, respectively. The kAE1 isoform is a truncated form of the eAE1 protein starting at Met-79 of eAE1 (Brosius-III et al., 1989). Although it is generally agreed that kAE1 localises to the basolateral surface of α-intercalated cells, there is also one report that kAE1 is found at the apical surface of β-intercalated cells (van Adelsberg, Edwards & Al-Awqati, 1993). There has also been one report of a novel alternate transcript of AE1 (nAE1), expressed in cardiomyocytes, that was characterised by a PCR mapping strategy (Richards et al., 1999) (Figure 1.5).

1.2.2.5.1.2 AE2

AE2 was originally isolated from the human erythroleukemic cell line K562 (Demuth et al., 1986) and subsequently from several other tissues (Alper et al., 1988; Kudrycki et al., 1990; Lindsey et al., 1990b). AE2 transcription can be initiated at three different points, generating alternatively spliced mRNAs, which encode three proteins that are N-terminally truncated at different places within the first 200 amino acids (Wang et al., 1996) (Figure 1.5). Northern blots have shown that AE2 is expressed in a wide variety of tissues. In humans, AE2 mRNAs were found in all tissue-derived cell lines examined (Demuth et al., 1986). Mouse AE2 mRNA has a similar broad tissue distribution (Alper et al., 1988) and rat AE2 was also present in all tissues examined, most abundantly in

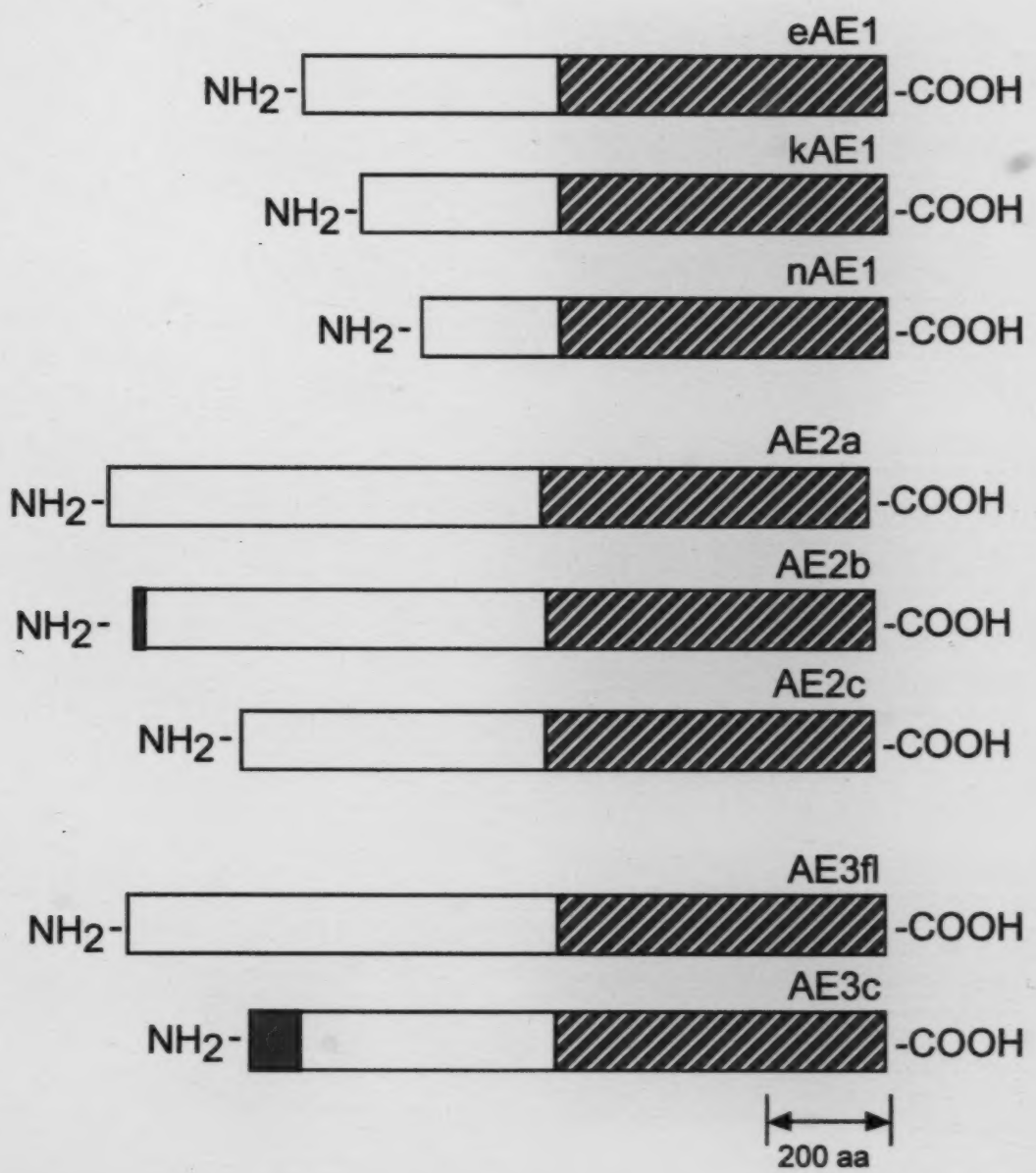


Figure 1.5 Schematic of spliced variants of AE proteins.

The striped portion represents the C-terminal transmembrane region and the open portion represents the N-terminal domain. The dark coloured box represents unique amino acids at the N-terminus. e-AE1 erythrocyte AE1, k-AE1 kidney AE1, nAE1 novel AE1

the stomach and portions of the GI tract (Kudrycki et al., 1990). AE2 is also found in the BLM of choroid plexus epithelia and plays a role in cerebrospinal fluid production (Lindsey et al., 1990a). Despite the presence of AE2 transcripts in most if not all tissues, expression at the protein level is more limited. This being said, AE2 is the most widely expressed AE isoform.

1.2.2.5.1.3 AE3

AE3 is predominantly found in excitable tissues such as brain (Kopito et al., 1989), heart (Linn et al., 1992) and retina (Kobayashi et al., 1994). AE3 mRNAs in the brain (full-length AE3) and heart (AE3c) are derived from different primary transcripts which originate at alternative promoters (Linn et al., 1992). The 5'-untranslated and the 5'-coding sequence of the full-length AE3 mRNA are contained within exons 1-6 and the corresponding sequences of the AE3c mRNA are contained within intron 6. Exons 7-23 encode sequences common to both full-length and cardiac AE3 (Linn et al., 1992). Human full-length AE3 is 1232 amino acids long and AE3c is 1034 amino acids, including the unique 73-amino-acid domain at the N-terminus (Figure 1.5) (Yannoukakos et al., 1994). Heart and retina both co-express the two different isoforms of AE3. AE3c is the most abundant AE protein expressed in the heart (Linn et al., 1995), whereas in the retina AE3c and full-length AE3 are expressed at similar levels, but in different retinal cell layers (Kobayashi et al., 1994).

1.2.2.5.2 AE structural features

Hydropathy analysis and sequence comparison of the AE gene family shows several structural features of the Cl^-/HCO_3^- exchangers. Figure 1.6 shows a topology model of AE1 determined experimentally (Reithmeier, Chan &

Popov, 1996; Tang et al., 1998). As with NHE, Cl^-/HCO_3^- exchangers possess two separate functional domains: a divergent N-terminal cytoplasmic domain (Steck, Ramos & Strapazon, 1976), involved in protein/protein interactions (Low, 1986), and a C-terminal membrane domain (Steck et al., 1976), which spans the lipid bilayer 12-14 times (Tanner, 1997) and is responsible for Cl^-/HCO_3^- exchange activity (Grinstein et al., 1978; (Lieberman & Reithmeier, 1988; Wainwright et al., 1989).

The AE cytoplasmic domain is the focal point for interactions between the plasma membrane and the underlying erythrocyte spectrin/ankyrin skeleton. The crystal structure of the cytoplasmic domain reveals that the cytoplasmic domain consists of 11 β-strands and 10 helical segments that can exist as a monomer, dimer and tetramer (Zhang et al., 2000). The cytoplasmic domain functions primarily as an anchoring site for other membrane-associated proteins. Some of these are ankyrin (Bennett & Stenbuck, 1980), protein 4.2 (Rybicki et al., 1993), protein 4.1 (Pasternack et al., 1985), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Rogalski, Steck & Waseem, 1989), phosphofructokinase (Jenkins, Madden & Steck, 1984), aldolase (Murthy et al., 1981), hemoglobin (Walder et al., 1984) and the protein tyrosine kinase $p72^{syk}$ (Harrison et al., 1994). Each of these interactions has important consequences with regard to erythrocyte structure and function including control of cell shape and flexibility (Low et al., 1991), as well as the regulation of both glucose metabolism (Low, Rathinavelu & Harrison, 1993) and ion transport (Malik, Sami & Watts, 1993) and ultimately in cell life span (Kannan, Yuan & Low, 1991). AE2

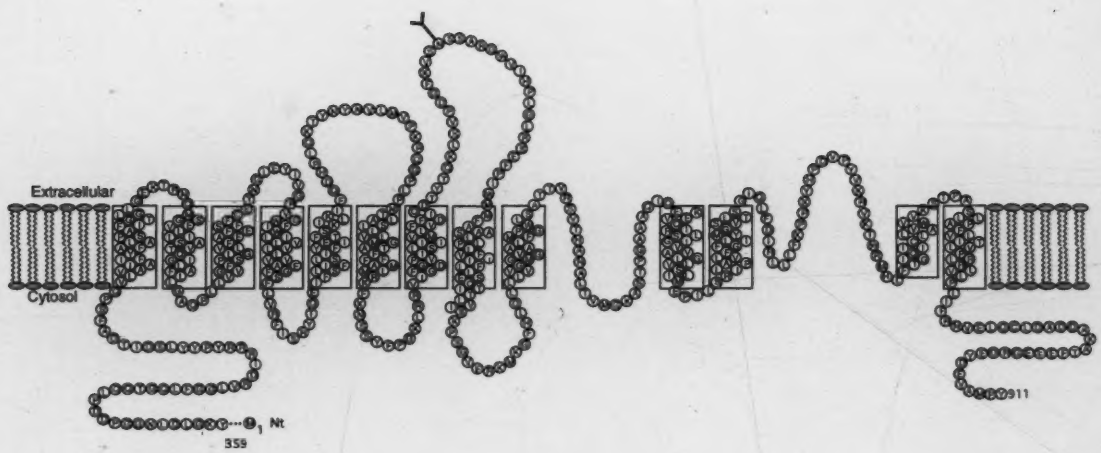


Figure 1.6 Topology model of AE1 based on experimental evidence.
The Y structure marks the position of N-linked glycosylation.

and AE3 also have large cytoplasmic domains, yet little is known about their structure and function.

1.2.2.5.3 Stoichiometry and kinetics

AE proteins exchange Cl^- for HCO_3^- in a 1:1 electroneutral anion exchange process (Jennings, 1982) that operates by a sequential ping-pong mechanism (Frohlich & Gunn, 1986; Jennings, 1989; Passow, 1986). This mechanism describes a protein that has a single exchange site able to exist in two states. In one state the anion-binding site is accessible from the intracellular surface of the membrane, while in the other state, the anion-binding site is accessible from the extracellular surface. During an exchange cycle, an anion binds to AE1 and induces a conformational change that causes the binding site to shift and face the other side of the plasma membrane. The bound anion diffuses away and leaves the binding site unoccupied. The site is then occupied by another anion, again causing a conformational shift, and the binding site returns to face the original side. In the absence of anions, the inability of the empty transporter to convert between the two states inhibits exchange via the AE protein because the complete cycle cannot occur unless an anion moves in either direction to return the transport proteins to its original state.

The ping-pong mechanism of exchange is supported by evidence that in the absence of a transportable anion on both sides of the membrane, transport will proceed only at an extremely low rate. The transport that does occur is known as "slippage" and is thought to be due to the rare conformational shift of the transport site from one side to the other, in an empty state. More support for the ping-pong mechanism comes from the fact that a high concentration of

transportable anions on one side of the membrane causes the binding site to be "recruited" to that side so that movement of the transport site is limited by the substrate at the lower concentration. Moreover, in the presence of a rapidly transported substrate on one side of the membrane and a poorly-transported substrate on the other, the transport rate is a function of the rate of transport of the slowly transported anion (Frohlich & Gunn, 1986; Jennings, 1989; Passow, 1986).

1.2.2.5.4 Anion specificity

AE proteins mediate the exchange of the physiological substrates Cl^- and HCO_3^- with a turnover rate of 5×10^4 per second at 37 °C, approximately one order of magnitude slower than a channel (Jennings, 1989). In addition, AE proteins can transport a range of other small inorganic and organic anions (Jennings, 1989). AE1 transports Br^- and F^- as rapidly as Cl^- and HCO_3^-, whereas I^- and HPO_3^{2-} are transported at a 10 fold slower rate. Divalent anions, such as SO_4^{2-}, are transported 10^4 times slower than Cl^-. This is thought to be due to the obligate electroneutrality of anion exchange, which means that a H^+ must be co-transported with SO_4^{2-} to neutralise the charge. AE can also facilitate the transport of small organic phosphates and organic anions such as N-(4-azido-2-nitrophenyl)-2-aminoethanesulphonate (NAP-taurine), although only at a very slow rate (Knauf et al., 1978).

1.2.2.5.4 Inhibition of AE proteins

AE1 was first identified as the erythrocyte anion exchanger, using the inhibitor DIDS (Cabantchik & Rothstein, 1974). Since then, the stilbenedisulphonate family of anion transport inhibitors has been used

extensively to study AE structure and function. The stilbenedisulphonate/AE1 interaction can be either covalent or non-covalent with binding affinities that range from 2 μM (DIDS) to 300 μM (DADS) (Cabantchik & Greger, 1992). Stilbenedisulphonates inhibit anion exchange on the protein's external face. Since the stilbenedisulphonate binding site is thought to overlap the anion binding site (Falke & Chan, 1986), identification of residues involved with stilbenedisulphonate binding is of great interest.

DIDS, the most commonly used covalently acting stilbenedisulphonate, contains two isothiocyano groups, which covalently react with deprotonated lysine amino groups. DIDS reacts with AE at neutral pH, with a stoichiometry of 1:1 (Lepke et al., 1976). The AE isoforms vary in their sensitivity to DIDS with inhibition of AE1 > AE3 > AE2 (Lee et al., 1991; Sterling & Casey, 1999).

An increase in AE1 thermal stability is associated with DIDS labeling of AE1 (Oikawa, Lieberman & Reithmeier, 1985) and DIDS labeling of AE1 in erythrocytes stabilises cells against pressure-induced haemolysis (Yamaguchi, Matsumoto & Kimoto, 1995). Other stilbenedisulphonates, DNDS and BIDS, can induce conformational changes in AE1 (Macara, Kuo & Cantley, 1983; Tang & Casey, 1999). Although it was not clear which regions of AE1 underwent conformational changes with BIDS (Macara et al., 1983), DNDS induced a conformational change in the regions of extracellular loops (EC) 3, 4 and 5 of AE1 (Tang & Casey, 1999).

There are other inhibitors of AE although they are not as commonly used as the stilbene disulphonates. Pyridoxal phosphate can be transported by AE1 and reacts covalently with a single lysine residue on AE1 in the presence of

$NaBH_4$, inhibiting AE transport activity (Kawano et al., 1988). The interaction of AE1 with eosinyl-5-maleimide occurs with a stoichiometry of 1:1 (Cobb & Beth, 1990) and results in complete inhibition of anion exchange in erythrocytes (Nigg & Cherry, 1979). As eosinyl-5-maleimide labeling blocks H_2DIDS labeling of AE1 it is thought that the eosinyl-5-maleimide binding site overlaps that of H_2DIDS (Cobb & Beth, 1990; Macara & Cantley, 1983).

Polyaminosterol analogs of the shark antibiotic squalamine display differential inhibition of AE1 and AE2 and are 8-fold more potent as inhibitors of AE2 than of AE1 (Alper et al., 1998). The oxanol, diBA(5)C4, however, is a >100-fold more potent inhibitor of AE1 than AE2 (Alper et al., 1998). AE-mediated anion exchange activity is also inhibited by 1,2-cyclohexanedione (Zaki, 1981) and phenylglyoxal (Bjerrum, Wieth & C. L. Borders, 1983; Wieth & Bjerrum, 1982).

In combination with $NaBH_4$, Woodward's reagent K converts the carboxyl sidechain of a glutamate residue (Glu 681) in the transport region of AE1 to a hydroxyl (Jennings & Al-Rhaiyel, 1988; Jennings & Anderson, 1987; Jennings & Smith, 1992). The glutamate residue 681 is conserved between AE1, AE2 and AE3 (Jennings & Smith, 1992). Conversion of the residue's sidechain from carboxyl to hydroxyl is associated with inhibition of monovalent anion transport and stimulation of divalent anion (SO_4^{2-}) transport (Jennings & Al-Rhaiyel, 1988). During Cl^--SO_4^{2-} exchange, sulphate is co-transported with a proton, supplied by E681 of AE1, to maintain electroneutrality (Milanick & Gunn, 1982; Milanick & Gunn, 1984). Mutation of E681 to serine removes the requirement for proton co-

transport by sulphate, thus stimulating divalent anion transport by AE proteins (Sekler, Lo & Kopito, 1995).

1.2.2.5.5 Regulation by pH

AE1 transports anions over a broad pH range (pH 5-11) (Alper, 1991; Kopito et al., 1989; Passow, 1986). AE2 on the other hand is steeply negatively regulated by acidic pH, consistent with its role in cellular acidification (Humphreys et al., 1994; Jiang et al., 1994). In CHOP cells (Chinese hamster ovary cells stably transfected with the polyoma large T antigen), variations in pH_i but not pH_o caused AE2 transport to be 20% active at pH 7.3 and approximately 8% active pH 6.9 (Jiang et al., 1994). In HEK 293 cells when both pH_i and pH_o were changed together, AE2 was 65% active at pH 7.3 and activity was reduced to 37% of maximum at pH 6.0 (Sterling & Casey, 1999).

Study of AE1/AE2 chimeric proteins assigned the distinct pH-regulatory functions to the N-terminal cytoplasmic domain and the C-terminal transmembrane domain of AE2 (Humphreys et al., 1997). The transmembrane domain site was named a "sensor "and the cytoplasmic domain site was named a "modifier" (Humphreys et al., 1997). Further studies have focused on the specific identity of the modifier site. N-terminal truncations have narrowed the required cytoplasmic region to residues between amino acids 328-347 and 391-510 (Alper, Chernova & Stewart, 2001). Continued investigations into these specific regions have identified sites that are involved in regulation of AE2 by pH_i alone and sites that are involved in regulation of AE2 by both pH_i and pH_o (Alper et al., 2001).

Changes in pH have also been shown to affect expression of AE2 in the choroid plexus. As assessed by Northern blots, six hours of alkalosis induced a 40% increase in AE2 mRNA levels in rats subjected to alterations in blood pH. In contrast, changes in osmolality did not affect AE2 mRNA (Keep et al., 1997).

Studies of AE3 produce conflicting results depending on whether pH_i, pH_o or both pH_i and pH_o are varied. When only pH_i was varied, AE3 was found to be negatively regulated by acidic pH being almost inactive below pH 7.3 (Lee et al., 1991). In that study however, Cl^-/HCO_3^- exchange was quantified by the rate of change of pH_i, measured by following the rate of pH_i recovery in cells made acidic or alkaline. Thus the variable under study (the effect of pH_i) varied constantly during measurement of transport. In another study, both pH_i and pH_o were simultaneously varied and pH values were clamped using the nigericin high potassium technique. Under these conditions the transport activity of both full-length and cardiac AE3 isoforms remained insensitive to changes in pH over a range of pH 6.0 - 9.0 (Sterling & Casey, 1999)(Chapter 3).

Measurement of AE activity in perfused ventricular myocytes showed that AE-mediated bicarbonate efflux increased as pH_i increased (Leem, Lagadic-Gossmann & Vaughan-Jones, 1999). The same investigators however have reported that, as pH_o increases, then AE-mediated bicarbonate transport decreases (Richard Vaughan-Jones, personal communication). The investigators have not yet determined which AE isoform is regulated in these studies of cardiomyocytes. However, if both pH_i and pH_o are changed at the same time the opposing changes in AE-mediated bicarbonate flux would effectively cancel each other out and the AE isoform responsible would appear to be unaffected by

changes in pH_i/pH_o. Previous studies have shown that the transport activities of AE1 and AE3 are unaffected when both pH_i and pH_o are varied together (Sterling & Casey, 1999). The transport activity of full-length AE3 however, is increased by an increase in pH_i when pH_o remains steady (Lee et al., 1991). Taken together these results suggest that the AE isoform that is differentially regulated by changes in pH_i and pH_o may be full-length AE3.

1.2.2.5.6 Regulation by kinases

AE2-mediated Cl^-/HCO_3^- exchange activity is activated 2-4 fold following short-term incubation with serum and the activation is reversible upon removal of the serum stimulus (Jiang et al., 1994). The stimulation of AE2 in the presence of serum is similar to the activation of endogenous Cl^-/HCO_3^- exchange activity by serum in vero cells (African green monkey kidney cells) (Tonnessen et al., 1990) although the AE isoforms expressed in vero cells has not been defined. Serum may alter AE2 pH_i sensitivity by causing the threshold value for activation of the transport protein to be shifted to a more acidic value (Tonnessen et al., 1990). Phorbol-12-myristate-13-acetate (PMA) stimulated vero cell AE transport activity in a similar manner to that observed with serum stimulation of AE2, suggesting that the AE transport activity in vero cells may be AE2, and therefore stimulation of AE2 by serum likely occurs in a PKC-dependant manner (Tonnessen et al., 1990). However in AE2-transfected HEK 293 cells, AE2 was not responsive to PMA (Alvarez et al., 2001). Alternatively, long-term serum starvation (12 hours) decreased AE2 transport activity significantly (~50%), while having no effect on AE3 (D. Sterling and J. Casey, unpublished observation).

Cardiomyocyte Cl^-/HCO_3^- exchange activity is also subject to regulation by angiotensin II and endothelin I through a PKC-dependent regulatory pathway (Camilion de Hurtado et al., 2000; Camilion de Hurtado et al., 1998). Investigation of AE3 isoforms expressed in HEK 293 cells suggests that full-length AE3 mediates the angiotensin II stimulated Cl^-/HCO_3^- exchange activity in cardiomyocytes. This stimulation occurs via direct phosphorylation by PKCε on Ser 67 of full-length AE3 (Alvarez et al., 2001). Interestingly incubation with the diacyl-glycerol mimetic, PMA, decreased AE3c transport activity (Alvarez et al., 2001), indicating differential regulation of the two isoforms due to their divergent N-termini (Figures 1.2 and 1.5). β-adrenergic and purinergic agonists also stimulate cardiac Cl^-/HCO_3^- anion exchange (Désilets, Pucéat & Vassort, 1994; Pucéat, Clément & Vassort, 1991).

The most important and best known hormonal system involved in the regulation of Na^+ levels is the renin-angiotensin-aldosterone system. Aldosterone is the physiological agonist of mineralocorticoid receptors and increases Na^+ reabsorption in the kidney. Aldosterone also stimulates Cl^-/HCO_3^- exchange activity in Madine-Darby canine kidney cells (Oberleithner et al., 1990) and rat neonatal cardiomyocytes (Korichneva et al., 1995b). However Na^+ depletion and aldosterone inhibit Cl^-/HCO_3^- in rat colon (Rajendran et al., 2000).

Tyrosine kinases have also been implicated in phosphorylation of AE1. A role for tyrosine kinases is suggested by the observation that tyrosine kinase inhibitors inhibit purinergic activation of anion exchange activity in cardiomyocytes (Puceat, Roche & Vassort, 1998). Also, exposure of erythrocytes to hypertonic conditions resulted in rapid phosphorylation of human AE1 on

tyrosine residues (Minetti et al., 1998). The level of AE1 tyrosine phosphorylation changes inversely with cell size as the cell swells and shrinks. The increase in phosphorylation during cell shrinkage was demonstrated to be specifically derived from an activation of tyrosine kinases (Minetti et al., 1998). No effect on AE transport function has been shown to result from hypertonically induced phosphorylation of AE1, although phosphorylation-dependent increase in membrane flexibility would enable a rapid passage of shrunken erythrocytes through the hypertonic passages in the kidney. Incubation with the tyrosine phosphatase inhibitor, orthovanadate, inhibited both full-length and cardiac AE3 transport activity by 20%, suggesting that tyrosine kinases inhibit the activity of AE3 isoforms (D. Sterling and J. Casey, unpublished observations).

1.2.2.6 Other bicarbonate transporters

In addition to the AE and NBC families of bicarbonate transport proteins, many other bicarbonate transport proteins have recently been cloned, revealing the presence of a bicarbonate transporter superfamily (Table 1.1). The recently cloned bicarbonate transporters are described below.

1.2.2.6.1 AE4

Initially cloned from rabbit β-intercalated cells, AE4 cDNA encodes a 955 amino acid membrane protein predicted to span the membrane 12 times (Tsuganezawa et al., 2001). Although named AE4, this bicarbonate transporter bears less amino acid sequence similarity to the AE family (34% identical to rabbit AE2) than to members of the NBC family (48%, 41% and 38% identical to human NBC-1, 2 and 3 respectively) (Tsuganezawa et al., 2001). Northern blots

indicate that AE4 is expressed mainly in the kidney, although small amounts were noted in the large intestine, small intestine and spleen. AE4 transport activity, assessed in *X. laevis* oocytes, showed that AE4 mediates Na-independent, DIDS sensitive Cl^-/HCO_3^- exchange and can also mediate Cl^-/base exchange in the absence of HCO_3^- (Tsuganezawa et al., 2001).

Human AE4 (BTR-1) was later cloned and found to share 84% identity with the rabbit protein. The BTR-1 cDNA encodes an 891 amino acid polypeptide and hydropathy analysis indicates 14 transmembrane spans and two predicted N-glycosylation sites on the third extracellular loop. SDS-PAGE analysis reveals two closely spaced bands with a molecular weight of ~90 kDa. The higher molecular weight band represents a glycosylated form and disappears when translation is carried out in the presence of the glycosylation inhibitor peptide, NLT (Parker, Ourmozdi & Tanner, 2001). Northern blots showed strong human AE4 signals in human kidney, salivary gland, testis, thyroid and trachea and weaker signals in cerebellum, oesophagus and mammary gland. Additionally, EST clones for AE4 were identified from human ovary and lung squamous cell carcinoma indicating that BTR-1 transcripts may be present at very low levels in these tissues (Parker et al., 2001). As with rabbit AE4, the human AE4 shares a higher amino acid sequence identity with NBC proteins than with AE proteins. Characterisation of the human AE4 transport activity indicates that it displays Na^+/HCO_3^- co-transport activity not Cl^-/HCO_3^- exchange activity (W. Boron and M Tanner, personal communication).

1.2.2.6.2 DRA

Human DRA, cloned from a colon subtraction library, is expressed in the normal colon but not in most adenocarcinomas, thus the term down regulated in adenoma (Schweinfest et al., 1993). Because expression was lost in colon neoplastic cells, it was originally speculated that DRA might be a tumor suppressor gene involved in colon carcinogenesis (Schweinfest et al., 1993). The protein product of the DRA gene is a membrane glycoprotein expressed in the duodenum, ileum, caecum, colon, seminal vesicle and sweat gland (Byeon et al., 1996; Haila et al., 2000). In mice, DRA expression is first seen in the luminal area of the midgut at embryonic day 16.5 and can be detected along the luminal layer of the developing small intestine by day 18.5. Colonic expression becomes evident by birth and in the adult mouse, DRA is found in the epithelial layers of the large and small intestines (Byeon et al., 1996).

DRA protein is most related in its proposed structure and amino acid sequence to the protein encoded by the human diastrophic dysplasia gene, DTSDT (60% similar, 33% identical) (Hastbacka et al., 1994) and to the rat liver SAT-1 protein (59% similar, 32% identical) (Bissig et al., 1994). Both DTSDT and SAT-1 are sulphate transport proteins and the similarity to these proteins would therefore suggest that DRA might also be a sulphate transport protein. Indeed, expression in oocytes resulted in Na^+ independent transport of both sulphate and oxalate that was sensitive to DIDS (Silberg et al., 1995).

Further characterisation of DRA function in *X. laevis* oocytes demonstrated that DRA can transport Cl^- (Moseley et al., 1999). When expressed in HEK 293 cells, mouse DRA mediated Na^+-independent electroneutral Cl^-/HCO_3^- exchange activity (Melvin et al., 1999). Cl^-/HCO_3^- exchange function in

the human colon and ileum has also been attributed to DRA (Melvin et al., 1999; Rajendran et al., 2000).

The anion specificity of DRA is relatively broad as evidenced by its ability to mediate the uptake of sulphate, oxalate and Cl^-/HCO_3^- exchange. In addition, while investigating Cl^-/HCO_3^- exchange in apical membrane vesicles from human proximal colon, it was noted that bromide, nitrate and acetate inhibited uptake of $^{36}Cl^-$ and suggested that the colonic Cl^-/HCO_3^- exchange protein, DRA, might also accept these ions as substrates (Mahajan et al., 1996). Taken together it appears that the physiological function of DRA may be not only the absorption of Cl^- from the intestine, but absorption of other anions as well.

A number of diseases are now recognised to result, at least in part, from defects in DRA. One of these, congenital chloride diarrhoea (CLD), is an autosomal recessive disorder of intestinal electrolyte absorption (Hoglund et al., 1998; Hoglund et al., 1996), which is rare worldwide but common in both Finland and Poland (Norio et al., 1971; Tomaszewski, Kulesza & Socha, 1987). CLD is caused by one of eight mutations in the DRA gene (SLC26A3) (Hoglund et al., 1998). In the ileum and colon NaCl absorption is mediated by the co-operation of Na^+/H^+ and Cl^-/HCO_3^- exchangers (Binder et al., 1987). Humans with CLD show defects in Cl^-/HCO_3^- exchange in the ileum and colon (Holmberg, Perheentupa & Launiala, 1975). In CLD, the defective HCO_3^- secretion leads to metabolic alkalosis and the acidification of intestinal content that further inhibits the absorption of Na^+ through NHE.

Another disease that DRA defects appear to contribute to is Cystic Fibrosis (CF), which is an autosomal recessive disease arising from inactivation

or mis-processing of a cAMP-sensitive Cl^- channel, known as the CF transmembrane conductance regulator (CFTR) (Rosenstein & Zeitlin, 1998). CF results in defective fluid and electrolyte secretion in secretory epithelia (Quinton, 1990; Welsh & Fick, 1987) impairing the respiratory, pancreatic, hepatobilliary and genitourinary systems (Rosenstein & Zeitlin, 1998). Recent studies have shown that the expression of DRA in both trachea epithelial cells and cultured pancreatic duct cells increased in the presence of active CFTR. This increase in expression of DRA is concurrent with an increase in Cl^-/HCO_3^- transport activity (Greeley et al., 2001; Wheat et al., 2000). The authors conclude that the HCO_3^- secretion defect in CF patients, results partly from the downregulation of the Cl^-/HCO_3^- exchange activity mediated by DRA.

Misregulation of DRA can not only be a cause but also a consequnce of some disease states. Intestinal inflammation downregulates DRA expression (Yang et al., 1998). Two animal models of colitis, the HLA-B27/β2M transgenic rat and an IL-1β knockout mouse were examined to determine if, like AE2 (Prieto et al., 1993), DRA expression is altered in an inflammatory state. Northern blots demonstrated that DRA mRNA was decreased in the transgenic rat and *in situ* hybridisation showed a reduction in DRA protein expression in both animal models (Yang et al., 1998). Although the mechanism by which DRA expression was diminished was not made clear, the fact that DRA expression in Caco-2 was down-regulated in the presence of IL-10, indicates that cytokines might play a role (Yang et al., 1998).

1.2.2.6.3 Pendrin

Pendreds' syndrome (OMIM no. 274600), first described by Vaughan Pendred in 1896, is a relatively common, autosomal recessive disorder characterised by deafness and goiter. The Pendred's syndrome gene (SLC26A4 or PDS) was identified by a positional cloning strategy (Everett et al., 1999) and encodes a protein named pendrin. Although pendrin is closely related to many sulphate transport proteins, it does not effectively transport sulphate, but rather functions as a Cl^-/iodide (Scott et al., 1999), Cl^-/formate (Scott & Karniski, 2000) and Cl^-/HCO_3^- transporter (Royaux et al., 2001; Soleimani et al., 2001).

Northern blot and RT-PCR analysis have identified pendrin expression in the thyroid, kidney and ear. *In situ* hydridisation established the expression pattern of pendrin in the developing auditory and vestibular systems (Everett et al., 1999). The expression pattern for pendrin places it in regions of the inner ear involved in endolymphatic fluid reabsorption, consistent with its function as a Cl^-/HCO_3^- exchanger.

Immunolocalisation studies of mouse, rat and human kidney detected pendrin expression on the apical surface of intercalated cells. Furthermore, pendrin was expressed exclusively in intercalated cells that express H^+-ATPase in both apical and basolateral membranes yet do not express AE1. Together these results indicate that pendrin is the apical anion transporter that mediates renal bicarbonate secretion in intercalated cells of the cortical collecting duct (CCD) that are not related to a-intercalated cells (Royaux et al., 2001). Another study of pendrin expression and function in rat kidney, found that the proximal tubule is highly enriched in pendrin mRNA (Soleimani et al., 2001). It is therefore likely that the physiological role of pendrin is both

chloride/bicarbonate and chloride/formate exchange in the CCD and proximal tubule.

1.2.2.6.4 K^+/HCO_3^- co-transporter

When squid axons are dialysed with an alkaline fluid containing K^+, pH_i increases from ~7.35 to ~8.0. When dialysis ceases, a rapid decrease in pH_i back to the physiological level (~7.35) occurs. The base efflux that produced this pH_i recovery was blocked by the combination of extracellular K^+ and CO_2/HCO_3^-, but not by either K^+ or CO_2/HCO_3^- alone. This led to the suggestion that the base efflux might be mediated by a novel K^+/HCO_3^- co-transporter (Hogan, Cohen & Boron, 1995b). The proposed presence of a K^+/HCO_3^- co-transporter in squid axon was further supported in a follow-up study. In this study it was noted fact that a substantial base influx occurs in squid axons when K^+ and CO_2/HCO_3^- were simultaneously introduced to the extracellular buffer, but not when either K^+ or CO_2/HCO_3^- alone was added (Hogan, Cohen & Boron, 1995a). Equivalent K^+/HCO_3^- co-transport activity has not yet been found in any species other than squid.

1.2.2.6.5 SAT-1

The mechanism for biliary excretion of sulphate was investigated in canalicular rat liver plasma membrane vesicles (cLPM) (Palacin et al., 1990). Incubation of cLPM vesicles in the presence of an inside-to-outside bicarbonate gradient stimulated sulphate uptake 10-fold compared with the absence of bicarbonate. Initial rates of this bicarbonate gradient-driven sulphate uptake were saturable with increasing concentrations of sulphate (apparent Km, approximately 0.3 mM) and could be inhibited by probenecid, N-(4-azido-2-

nitrophenyl)-2-aminoethylsulphonate, acetazolamide, and DIDS. Measurement of sulphate uptake in bicarbonate-containing and bicarbonate-free buffers indicated the presence of a sulphate (oxalate)-bicarbonate anion exchange system in rat canalicular liver plasma membranes, which supports the suggestion that the presence of a sulphate (oxalate)-bicarbonate anion exchange system might play an important role in bile acid-independent canalicular bile formation (Palacin et al., 1990).

The rat canalicular sulphate/bicarbonate transport protein was later cloned by an expression cloning strategy in *X. laevis* oocytes (Bissig et al., 1994). The cloned transporter, called sat-1, is the first identified member of the SLC26 gene family. It is a 703 amino acid membrane protein containing 12 putative trans-membrane segments and three potential glycosylation sites. Northern blot analysis shows the presence of sat-1 in the kidney, skeletal muscle and brain (Bissig et al., 1994).

1.2.2.6.6 PAT-1

Using a homology approach combined with RACE-PCR, the gene SLC26A6 was identified in humans. The protein product of this gene is called PAT-1 and Northern blot analysis indicates the presence of PAT-1 in the kidney, pancreas, heart, skeletal muscle, placenta, intestine and liver. Low, but detectable levels of PAT-1 were also found in the brain and lung (Waldegger et al., 2001). Immunohistochemical studies localised PAT-1 to the brush border membranes of the villus cells of the duodenum (Wang et al., 2002) and expression in *X. laevis* oocytes demonstrated that PAT-1 functions as a Cl^-/HCO_3^-

exchanger (Wang et al., 2002). Therefore PAT-1 appears to be an apical Cl^-/HCO_3^- in the intestine.

1.3 Cellular functions affected by intracellular pH

The selective permeability of the plasma membrane to ions plays a central role in the generation of the cellular membrane potential and in the regulation of pH_i and volume. As summarised in Figure 1.7, pH_i modulates many diverse cellular events including metabolism, ionic conductances, Ca^{2+} homeostasis and cell division (Orchard & Kentish, 1990). The physiologic importance of pH_i is underlined by clinical manifestations of pH abnormalities such as changes in excitability of nerve and muscle cells. For example, acidosis represses the nervous system while alkalosis overexcites first the peripheral nervous system and later the central nervous system. The concentration of H^+ ions also exerts a marked influence on enzymatic activity where slight deviations in pH_i may severely alter enzymatic function.

Glucose metabolism results in the production of CO_2 under aerobic conditions and lactic acid under anaerobic conditions. Overall oxidative metabolism acidifies the cell. However, it is not only metabolism that affects the pH_i of a cell. The reverse is also true where pH_i affects cellular metabolism. For example, pH affects the charge on ionisable groups within proteins thereby changing protein conformation and consequently the activity of key metabolic enzymes. Phosphofructokinase, a glycolytic enzyme that converts fructose 6-phosphate to fructose 1,6-diphosphate, has maximal activity in the normal physiological range (7.0-7.2). However, as pH decreases, so too does the activity of this metabolic enzyme. Thus, maintenance of an appropriate pH_i maximises

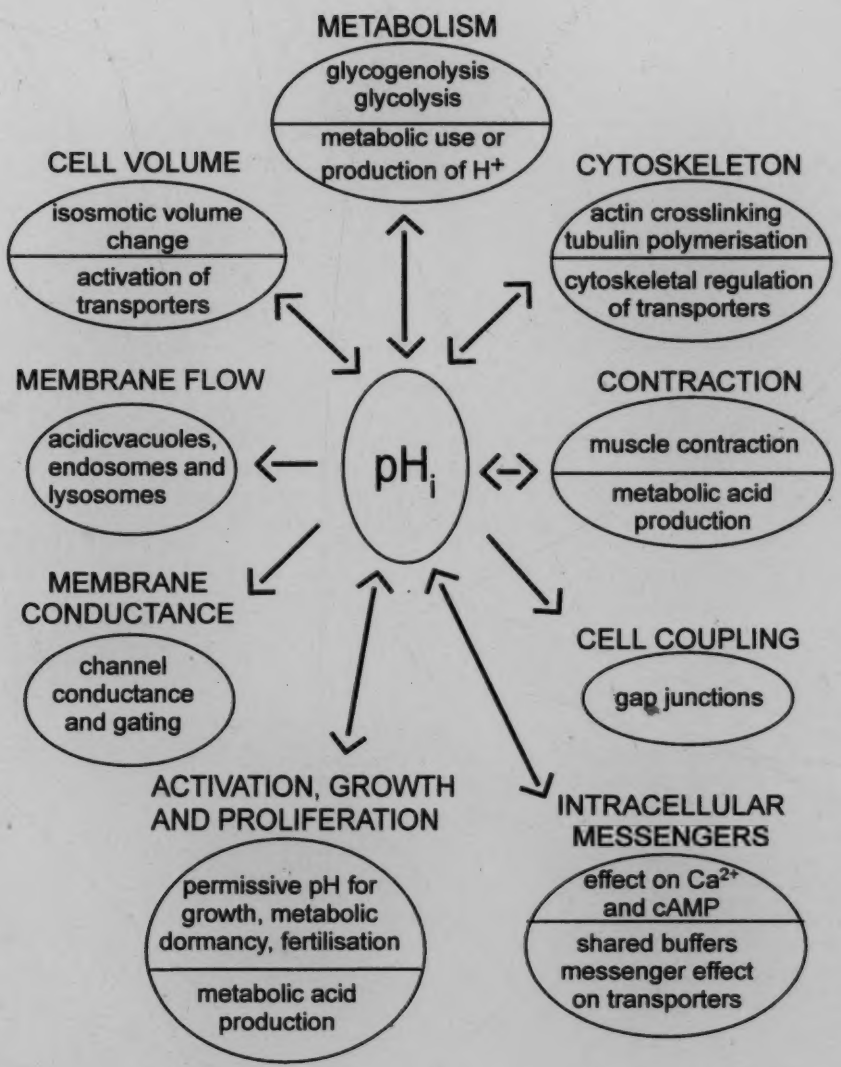


Figure 1.7 Summary of cellular processes affected by changes in pH_i.
Double headed arrows indicate that the pH_i can affect the cellular function and the process can affect pH_i. Single headed arrows suggest that while pH_i affects the cellular processes, the cellular processes probably do not affect pH_i. The top half of the circle indicates the cellular process affected by changes in pH_i and the bottom half indicates the mechanism by which the process can affect pH_i.

enzymatic activity, while the modulation of pH_i provides a mechanism for regulation of cellular metabolism. Changes in pH_i can also affect the cytoskeleton. In some cells alkalinisation increases actin cross-linking whereas in others cross-linking is decreased. Alkalinisation can also cause de-polymerisation of tubulin and disaggregation of microtubules within the cell (Putnam, 1998).

In cardiac muscle, both Ca^{2+} homeostasis and Ca^{2+} sensitivity of the myofilaments that closely regulate contraction are affected by changes in pH_i (Orchard & Kentish, 1990). Acid pH_i closes gap junction conductance and thereby blocks electrical coupling (Ek-Vitorin et al., 1996; Hermans et al., 1995). Therefore to maintain proper mechanical and electrical function, it is critical that a cardiac cell possesses mechanisms by which pH_i can be successfully regulated and maintained at a standard physiological level.

Changes of pH_i can affect levels of intracellular signaling molecules such as Ca^{2+} and cAMP. The effect of these pH_i changes on Ca^{2+} can be either direct or indirect. A decrease of pH_i can directly reduce Ca^{2+} entry across the plasma membrane as well as activate mitochondrial Ca^{2+}/H^+ exchange. Activation of Ca^{2+}/H^+ exchange, where protons are transported into the mitochondria in exchange for Ca^{2+}, results in H^+ sequestration in the mitochondria and a consequent increase in cytoplasmic Ca^{2+}. Moreover, many molecules that buffer H^+ ions also bind and thus buffer Ca^{2+} ions. Therefore, depending on relative affinities, an elevation of cytoplasmic $[H^+]$ can displace Ca^{2+} ions from intracellular buffer sites and increase intracellular Ca^{2+} levels.

Cl^-/HCO_3^- anion exchange proteins and Na^+/H^+ transport proteins transport Na^+ and Cl^- across the plasma membrane in exchange for a proton equivalent (HCO_3^- or H^+, respectively), thus regulating pH_i. Because the transported H^+ equivalent is buffered it is osmotically invisible. However the movement of osmotically invisible H^+ equivalents is accompanied by the movement of osmotically active Na^+ or Cl^-. AE and NHE often work in concert to cause a net influx of NaCl that is invariably accompanied by the movement of water leading to cell swelling. Activation of NHE and AE by changes in pH_i can therefore lead to an increase in cell volume (reviewed in (Putnam, 1998)). PKC dependant co-activation of NHE and AE in cardiomyocytes is implicated in cardiac hypertrophy (Alvarez et al., 2001; Perez et al., 1995). Activation of NHE causes accumulation of intracellular Na^+ which in turn impairs or reverses NCX activity (Cross et al., 1998). The consequent increase of Ca^{2+} causes a hypertrophic response while also stimulating PKC. This provides positive feedback for NHE and AE activation causing an ever increasing hypertrophic response (Karmazyn, 2001).

1.4 Intracellular pH regulation in cardiomyocytes

Intracellular buffers and H^+ equivalent transport proteins present in the plasma membrane (Figure 1.3) mediate regulation of pH_i in cardiac cells. These systems maintain pH_i in cardiac muscle at 7.0 - 7.2. The membrane proteins are sensitive to pH and will aid cells in recovery from an acid or alkali load. A clearer understanding of how these mechanisms function in response to an acid load is of great importance, as there is high mortality associated with ischaemic acidosis following a myocardial infarction.

NHE1 has deleterious effects on cardiac function following pH_i recovery (Figure 1.2). NHE1 contributes to the recovery of myocyte pH_i following an acid load through it's ability to extrude protons (Blatter & McGuigan, 1991). The extrusion of protons however, occurs in conjunction with an influx of sodium. AS explained earlier, accumulation of intracellular Na^+, which impairs the function of NCX by destroying the inward-directed Na^+ gradient. This leads to either a decrease in removal of intracellular Ca^{2+} or reversal of Na^+/Ca^{2+} exchange activity (Cross et al., 1998), the consequences being a rise in intracellular Ca^{2+} concentration and an upset in both Ca^{2+} homeostasis and Ca^{2+} sensitivity of myofilaments (Korichneva et al., 1995a). Excess Ca^{2+} can also precipitate into mitochondria causing cell necrosis (Korichneva et al., 1995a). Thus, the cellular damage that occurs from ischaemic acidosis is not actually due to the acidic load produced during the ischaemic phase, but results from reperfusion-associated Na^+ accumulation. Ironically, it is a mechanism that a cardiac cell utilises to recover from an acid pH_i that contributes to the cellular damage.

AE proteins, on the other hand, exchange Cl^- for HCO_3^- across the plasma membrane to regulate pH_i. The AE-mediated bi-directional movement of HCO_3^- suggests that AE proteins can either acidify or alkalinise cardiomyocytes. Following an ischaemic episode recovery of pH_i of acidotic cells by AE would occur in a Na^+ independent manner. AE-mediated Cl^-/HCO_3^- exchange activity would therefore contribute to recovery of pH_i without contribution to the toxic Na^+ overload that occurs following NHE and NBC activation.

Vandenberg and colleagues provided an elegant study describing mechanisms of pH_i recovery following global ischaemia of ferret hearts (Vandenberg, Metcalfe & Grace, 1993). Recovery of the ischaemic heart was investigated in both HCO_3^--containing and HCO_3^--free buffers containing various inhibitors. This enabled the determination of the contribution of each kind of transport protein to the recovery of pH_i in cardiomyocytes. If the role of AE proteins in pH_i regulation is taken into consideration, the contribution of each protein as a percentage of total recovery is given in Table 1.2. It is clear that although HCO_3^--dependent mechanism proteins provide the largest contribution, monocarboxylate transport provides the greatest contribution of a single transport mechanism to the recovery of pH_i.

The contribution of AE proteins to recovery of pH_i in ischaemic cardiomyocytes makes it evident that a clear understanding of AE transport activity in cardiomyocytes, particularly at low pH_i is imperative to determine the role of AE proteins. Definition of the AE transport processes in cardiomyocytes may provide a means for therapeutic manipulation of AE proteins.

1.5 Measurement of intracellular pH

The study of intracellular pH and its regulation was enabled by the development of the first reliable pH-sensitive microelectrode, called the recessed-tip microelectrode (Thomas, 1974). The recessed-tip microelectrode gave some of the first continuous, reliable measurements of intracellular pH and was used for over two decades in pH_i studies.

A number of biochemical techniques have now been devised to measure pH_i and changes in pH_i. One approach relies on the partition of weak bases or

Table 1.2 Table of the contribution of each transporter found in the heart to recovery from global ischaemia of a ferret heart.

Transporter	Contribution to pH_i recovery from ischemia (%)
Na^+/H^+ antiporter	19
Na^+/HCO_3^- co-transporter	15
Monocarboxylate/H^+ co-transporter	34
Cl^-/HCO_3^- exchanger	32

Recalculated from Vandenberg et al., 1993

weak acids across the membrane. These may be radioactive (dimethyloxazolidinedione or benzoic acid) or fluorescent (acridine orange and 9-aminoacridine). The technique however, requires knowledge of the intracellular water space and its time resolution is limited.

The classical approach for monitoring pH_i-regulating mechanisms in a cell is to load cells with OH^- or H^+ and observe recovery of pH_i to base line. A technique widely used to achieve this is the ammonia pulse technique (Boron & De Weer, 1976). Alternative techniques incorporate dyes that undergo changes in fluorescence upon reversible binding of H^+ ions. Spectrofluorometric measurements of the fluorescence of pH-sensitive probes such as pyranine (Damiano et al., 1984) or derivatives of fluorescein (Rink, Tsien & Pozzan, 1982) are now commonly used to measure pH_i. Pyranine is a permanently charged molecule. Therefore it does not cross membranes, and is useful only for work with vesicles. Biscarboethylcarboxyfluorescein (BCECF) is the most effective derivative of fluorescein (Figure 1.8). Like pyranine, BCECF is a membrane impermeable charged dye. However, BCECF can be rendered neutral by the addition of acetoxymethyl ester groups (BCECF-AM). In this uncharged form, the dye rapidly permeates through the plasma membrane. Once inside the cell BCECF-AM is rapidly hydrolysed by intracellular esterases, leaving it charged and trapped inside the cell.

An excitation spectrum can be obtained by exciting BCECF with light of a range of wavelengths (300-600 nm) and recording the fluorescence emission at 535 nm. The excitation spectrum for BCECF reveals that when the dye is excited at 440 nm its fluorescence is insensitive to changes in pH_i, whereas the dye is

very sensitive to changes in pH_i when excited at 500 nm. Fluorescence values of BCECF after excitation at both of these wavelengths provide a ratiometric reading of changes in fluorescence as a result of changes in pH_i. The fluorescence ratio provides an internal control that will correct for photobleaching or dye leakage (Rink et al., 1982).

The polyether ionophore, nigericin, couples the K^+ and H^+ gradients across the plasma membrane and when used in the presence of high K^+, can clamp pH_i at a known value to assist in calibrating fluorescence signals (Thomas et al., 1979). This can be used to convert the fluorescence ratio of BCECF to a measurement of pH_i.

The fluorescent dyes, BCECF and SPQ (6-methoxy-N-(3-sulphopropyl)quinolinium), provide an effective means to monitor AE transport activity. By changing the chloride concentration in the extracellular perfusate buffer either an inward or outward directed Cl^- gradient can be imposed on the cell. In AE-transfected cells, AE-mediated flux of Cl^- across the membrane is coupled to HCO_3^- flux. The pH sensitivity of BCECF allows for indirect measurement of changes in pH_i associated with AE-mediated HCO_3^- flux and the chloride sensitivity of SPQ provides a means to monitor AE-mediated Cl^- flux. Experimentally, anion transport activity of AE isoforms can be obtained by monitoring changes in fluorescence associated with AE-mediated HCO_3^- flux. This in turn provides a mechanism to observe changes in AE transport activity following stimulation or inhibition by extrinsic factors.

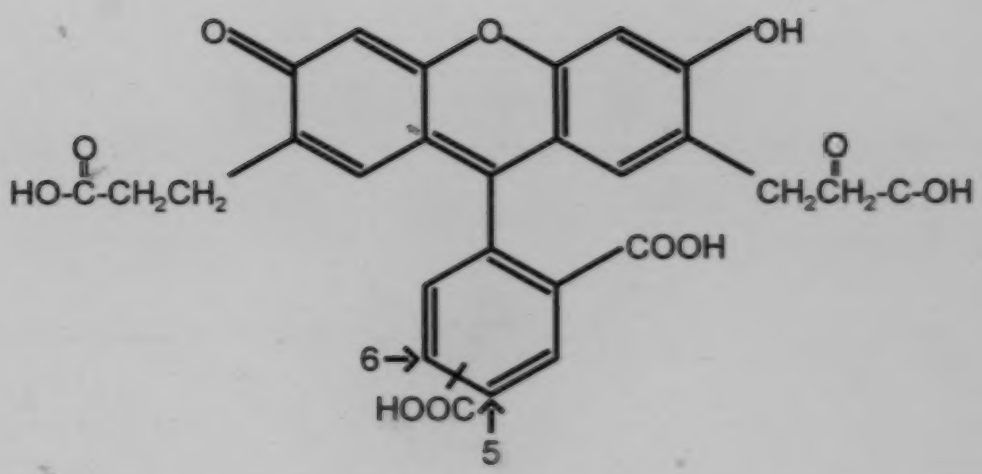


Figure 1.8 Chemical structure of the pH-sensitive fluorescent probe, BCECF.

1.6 Carbonic anhydrases

Carbonic Anhydrases (CA) are a family of zinc metalloenzymes (EC 4.2.1.1) that catalyse the hydration/dehydration of CO_2/HCO_3^-. CA is a ubiquitous protein found in all animals and photosynthesising organisms examined for its presence (Lindskog, 1997). Functionally CA activity is coupled to bicarbonate transport as illustrated by the cellular mechanism of gastric acid secretion (Figure 1.9).

1.6.1 Cloning and tissue distribution

14 mammalian CA isoforms have been identified to date differing in tissue distribution, catalytic activity and sensitivity to inhibitors (Kivela et al., 2000a; Mori et al., 1999; Tureci et al., 1998). CAI, II, III, VII are cytosolic isoforms. CAI is mainly found in erythrocytes and works in concert with CAII to facilitate the removal of the waste product, CO_2, from the body. Although expressed at a higher level than CAII in the erythrocyte, CAI has relatively slow catalytic activity (2×10^5 sec^{-1} at 25 °C) and is inhibited at physiological levels of chloride. Thus CAII provides the majority of CA activity in the erythrocyte. CAII has the widest distribution of all the CA isoforms and is located in the cytosol of most tissues (Sly & Yu, 1995). CAII is also the most catalytically active isoform, with a CO_2 hydration rate of 1×10^6 sec^{-1}. CAII is required in stomach acid secretion (Figure 1.9), bone resorption, urine acidification and aqueous humor formation (Sly & Yu, 1995; Whyte, 1993). CAIII is expressed in the cytosol of slow twitch red skeletal muscle fibres and adipocytes (Lindskog, 1997), where it assists in the diffusion of CO_2 from the muscle to capillary beds. CAIII is a low activity CA with a CO_2 hydration rate of 8×10^3 s^{-1} (Sly & Yu, 1995).

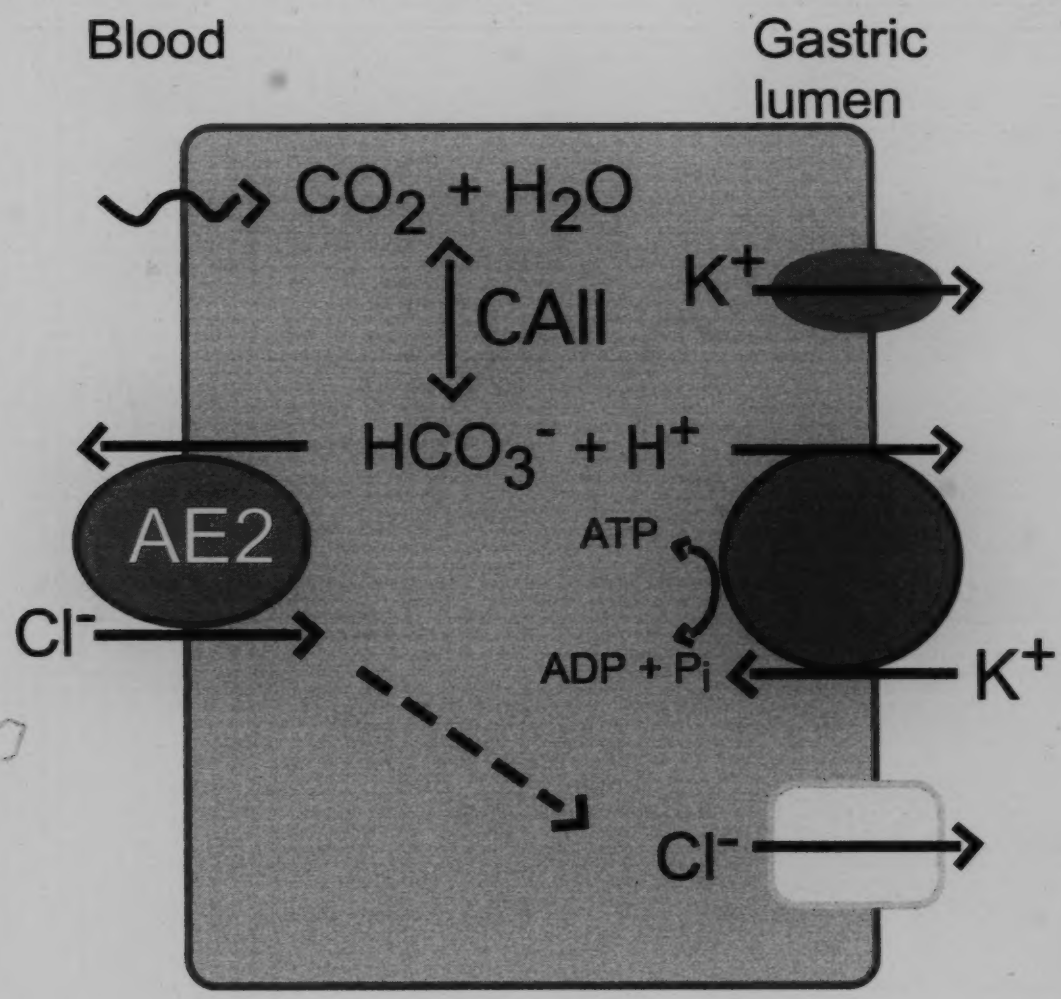


Figure 1.9 Gastric acid secretion.

The stomach's parietal cells actively secrete H^+ and Cl^-. The secreted H^+ is generated along with HCO_3^- during the hydration of CO_2 by carbonic anhydrase. Cl^- is transported into the cells via AE2 in exchange for HCO_3^-.

CAIV, IX, XII and XIV are membrane-associated CA isoforms. CAIV is anchored extracellularly to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (Waheed et al., 1992) and localises in lipid rafts. Northern blots, immunohistochemical and functional studies localised CAIV expression to the heart, lung, kidney, brain, retina and erythrocyte (Brion et al., 1994; Ghandour et al., 1992; Hageman et al., 1991; McKinley & Whitney, 1976; Sender et al., 1998; Sly et al., 1985; Tong et al., 2000; Whitney & Briggle, 1982; Wistrand et al., 1999; Wistrand & Knuuttila, 1989). As CAIV is linked extracellularly, it hydrates CO_2 in the extracellular space as opposed to the cytosol with a catalytic activity of $8x10^5$ s^{-1} (Baird et al., 1997).

CAIX and XII contain integral transmembrane segments. These isoforms have been associated with oncogenesis by acidifying the tumor microenvironment and thus facilitating tumor growth. Although over-expressed in cancerous tissues, these isoforms are also expressed in normal tissue. Both isoforms are associated with renal cell carcinoma (Parkkila et al., 2000; Tureci et al., 1998) and pancreatic tumors (Kivela et al., 2000b). CAXII expression is also associated with colon and lung cancer (Parkkila et al., 2000; Tureci et al., 1998; Vermylen et al., 1999).

CAXIV is the most recently identified membrane-associated CA isoform (Mori et al., 1999). Initially isolated from murine kidney CAXIV is also expressed in heart skeletal muscle, brain, lung and liver (Mori et al., 1999). CAXIV was recently found in human brain on neuronal membranes (Parkkila et al., 2001). CAXIV catalytic activity and sensitivity to inhibition by sulphonamides have not yet been determined.

CAVI is a secreted isoform found in saliva where it is postulated to regulate pH by converting HCO_3^- and H^+ to CO_2 and H_2O when the oral cavity becomes too acidic. CAVI secretion correlates with saliva secretion rates and amylase activity but not to changes in pH or buffer capacity pH (Kivela et al., 1997).

CAV is found in the mitochondria of mammalian liver. A role for CA in mitochondria was suggested when it was realised that mitochondria are impermeant to HCO_3^- ions and that HCO_3^- is required by pyruvate carboxylase in these organelles for gluconeogenesis and by carbamoyl phosphate synthase I for detoxification of ammonia in liver (Dodgson & Forster, 1986a; Dodgson & Forster, 1986b). CAV has a catalytic activity around 20 fold lower that CAII (Heck et al., 1996; Heck et al., 1994).

1.6.2 Inhibition of carbonic anhydrase

Sulphonamides, such as acetazolamide, methazolamide, ethoxzolamide and dichlorophenamide, contain an SO_2NH_2 moiety that binds to the Zn^{2+} ion within that carbonic anhydrase active site, and thus inhibits CA enzymatic activity. These compounds have been used for over forty years in the systemic treatment of open-angle glaucoma (Maren et al., 1983). Uncomfortable and debilitating side effects arising from systemic administration led to the development of carbonic anhydrase inhibitors with topical activity, such as dorzolamide and brinzolamide (Palmberg, 1995). To achieve the water solubility required for effective topical action of these compounds their hydrochloride salts are used (Palmberg, 1995). Such solutions have a relatively acidic pH (pH 5.5) and cause eye irritation accompanied with blurred vision (Scozzafava et al.,

2002). The clinical importance of topical carbonic anhydrase inhibitors has spurred a wave of research in the synthesis and evaluation of new carbonic anhydrase inhibitor derivatives that have reduced side effects. Reaction of polyamino-polycarboxylic acid with aromatic/heterocyclic sulphonamides produces water soluble, effective, topical carbonic anhydrase inhibitors (Scozzafava et al., 2002). Being water soluble, these compounds produce a topical carbonic anhydrase inhibiting agent of neutral pH that is devoid of the serious side effects associated with other compounds. Many other sulphonamide derivatives have been synthesised in the last few years, all of which show promising results in the treatment of glaucoma (Casini et al., 2001; Chazalette et al., 2001; Masereel et al., 2002; Mincione et al., 2001; Scozzafava et al., 2000a; Scozzafava et al., 2000b; Scozzafava et al., 2001a; Scozzafava et al., 2002; Scozzafava et al., 2001b; Scozzafava & Supuran, 2002).

The development of membrane-impermeant as well as membrane-permeant carbonic anhydrase inhibitors have helped to delineate the contributions of cytosolic and extracellular membrane bound CA isoforms. Benzolamide was the first membrane-impermeant CA inhibitor synthesised (Schwartz, 2001). Others include p-fluorobenzyl-aminobenzolamide (Tsuruoka et al., 2001), dextran-bound inhibitors (Lucci et al., 1983) and F-3500, an aminobenzolamide coupled to polyoxyethylene bis(acetic acid) (Matsui et al., 1996).

Some sulphonamides also inhibit Cl^-/HCO_3^- exchange by interacting with the HCO_3^- transport system. Movement of HCO_3^- across the membrane is facilitated by interaction of HCO_3^- with a component of the membrane or

transport protein. This interaction requires a three-point attachment involving three O_2 atoms in the substrate with the complementary loci on the transport protein. The sulphonamide group SO_2NH_2 can also satisfy the requirement for three point attachment because the electronegativity of N is similar to that of O (Cousin, Motais & Sola, 1975). Interestingly, in a study of 26 sulphonamide carbonic anhydrase inhibitors, acetazolamide was the only sulphonamide that did not inhibit Cl^-/HCO_3^- exchange directly (Cousin & Motais, 1976).

1.6.3 Carbonic anhydrase and bicarbonate transport proteins

Cardiomyocytes contain no cytosolic carbonic anhydrase. They do however express CAIV (Sender et al., 1998). In this case intracellular conversion of CO_2 to HCO_3^- would be too slow to keep up with metabolic CO_2 production. Since CAIV is present extracellularly, CO_2 that diffuses freely out of the cell will become rapidly converted to HCO_3^- as illustrated in figure 1.10.

As mentioned previously, during an ischaemic episode blood flow to cardiac tissue is greatly impaired. As metabolism continues anaerobically, acidic waste builds up in the cell. Upon reperfusion, the cardiomyocyte employs several mechanisms to recover pH_i to a normal physiological level. The action of NHE and NBC both cause Na^+ influx, resulting in the Na^+ overload described earlier. The presence of extracellular CA enables the extracellular concentration of HCO_3^- to increase substantially, creating an inward HCO_3^- gradient. This could permit the AE proteins to bring HCO_3^- into the cell in exchange for Cl^-, thus alkalinising the cell.

As with MCT there are two isoforms of AE3 found in the heart, suggesting that they have different functions or are differentially localised or

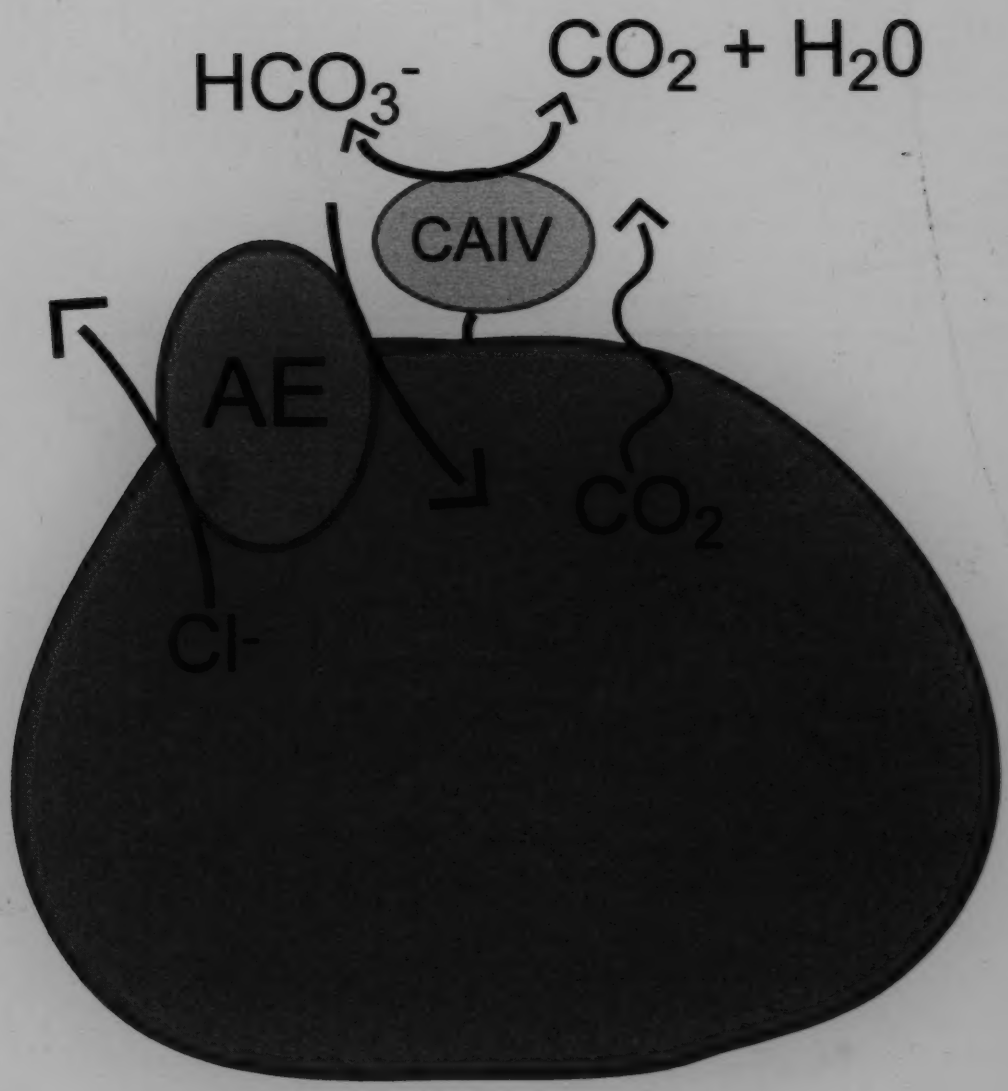


Figure 1.10 Cardiomyocytes express CAIV, an extracellular CA isoform.
Therefore rapid hydration of CO_2 occurs in the interstitial space as opposed to the cytosol. Rapidly produced extracellular HCO_3^- is transported into the cardiomyocyte via AE-mediated Cl^-/HCO_3^- exchange activity.

regulated. Therefore, at resting pH, one isoform may be active while the job of recovering to physiological pH from an acid load lies with the other isoform. Elucidating the contribution of each AE isoform to pH_i regulation in cardiomyocytes is vital to fully understand the mechanisms for recovery of pH_i available following an ischaemic episode.

Human erythrocytes express CAI, CAII and CAIV, the majority being the lesser active CAI (Maren, 1967; Wistrand et al., 1999). The turnover rate of CAII (10^6/s) and the fact that CAI activity is inhibited by physiological concentrations of Cl^- make it evident that CAII accounts for the majority of carbonic anhydrase activity in human erythrocytes.

Several lines of evidence show an interaction between CAII and AE. Binding of erythrocyte membranes to CAII increases CAII enzymatic activity (Parkes & Coleman, 1989), indicating an interaction between CAII and an erythrocyte membrane protein. CAII can be co-immunoprecipitated with solubilised AE1 and lectin-mediated agglutination of AE1 in erythrocytes lead to a similar redistribution of CAII (Vince & Reithmeier, 1998). Finally a sensitive microtiter assay showed that CAII but not CAI can bind the C-terminus of AE1 (Vince & Reithmeier, 1998). Truncation and point mutations of the AE1 C-terminus identified the binding site of CAII in AE1 as the acidic motif LDADD (aa 886-890) (Vince & Reithmeier, 2000). This places CAII immediately adjacent to the lipid bilayer in an ideal position for hydration of incoming CO_2. Binding of AE1 and CAII is pH dependant (Vince & Reithmeier, 1998), indicating an interaction between the acidic LDADD motif of AE1 with a basic region of CAII. Truncation and mutagenesis of the basic N-terminal region of CAII showed that

the AE1 binding site in CAII resides there (Vince, Carlsson & Reithmeier, 2000). Replacing basic residues in the N-terminus of CAII with the equivalent residues of CAI resulted in a loss of AE1 binding as did truncation of the N-terminus. Modification of the CAII N-terminus however, did not impair enzymatic activity. This suggests that the function of this basic N-terminal domain is to bind CAII to AE1, or other proteins with similar acidic binding motifs (Vince et al., 2000).

1.7 Overview of thesis

The importance of the AE proteins in pH_i regulation and bicarbonate transport underscores the need for further investigation of the regulation of AE proteins. The aim of this thesis was to understand some of the mechanisms that regulate the Cl^-/HCO_3^- exchange activity of AE1, AE2 and AE3. The studies presented here characterised the Cl^-/HCO_3^- exchange activity of AE proteins and the regulation of their transport activity by changes in pH and interaction with carbonic anhydrase. The findings reported here contribute to our understanding of regulation of AE transport activity by changes in pH and define the functional impact of the CA/AE interaction and the role this plays in accelerating AE transport activity.

Chapter 2

Materials and Methods[1]

[1]**Portions of this chapter have been previously published and are reproduced with permission from:**

Sterling, D., Alvarez, B. V. and Casey, J.R. (2002) The extracellular component of a transport metabolon: Extracellular loop 4 of the human AE1 Cl^-/HCO_3^- exchanger binds carbonic anhydrase IV, submitted to *J. Biol. Chem.* © The Biochemical Scoiety

Sterling, D., Brown, N. and Casey, J. R. (2002) The Functional and Physical Relationship Between the Downregulated in Adenoma Bicarbonate Transporter and Carbonic Anhydrase II, submitted to *Am. J. Physiol.*

Sterling, D., Reithmeier, R.A.F. and Casey, J.R. (2001) A Transport Metabolon: Functional Interaction of Carbonic Anhydrase II and Chloride/Bicarbonate Exchangers. *J. Biol. Chem.* **276** (51), 47886-47894

Sterling, D., Reithmeier, R.A.F. and Casey, J.R. (2001) Carbonic Anhydrase: In the Driver's Seat for Bicarbonate Transport. *J. Pancreas* **2** (4), 165-170

Sterling, D. and Casey, J.R. (1999) Regulation by Intracellular pH and Transport Activity of AE3 Cl^-/HCO_3^- Exchange Proteins. *Biochemical J.*, **344** (1), 221-229 © The Biochemical Scoiety

Materials and methods

2.1 Materials and Methods

ECL® Enhanced Chemiluminescence reagent and hyperfilm, donkey anti-rabbit IgG conjugated to horseradish peroxidase, the pGEX-6p-1 and pGEX-5X-3 expression vectors, the 5′ pGEX sequencing primer gggctggcaagccacgtttggtg, biotinylated anti-(rabbit IgG), glutathione Sepharose 4B and peroxidase labeled biotin/avidin where from Amersham–Pharmacia biotech (Quebec, Canada). Poly-L-lysine, o-phenylenediamine dihydrochloride and nigericin were from Sigma–Aldrich Canada (Oakville, Canada). pcDNA3.1 was from Invitrogen (Carlsbad, CA, USA). Molecular probes, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM) was from Cedarlane laboratories ltd. (Ontario, Canada), and 6-methoxy-n-(3-sulphopropyl)quinolinium (SPQ) was from Toronto Research Chemicals (Toronto, Ontario, Canada). Glass coverslips and Corning Life Sciences 96 well microtiter plates were from Fisher Scientific products (Nepean, Canada). Sulpho-NHS-SS-biotin and streptavidin–agarose were from Pierce chemical company (Rockford, IL, USA). Sheep anti-(human carbonic anhydrase II antibody was from Serotec (Raleigh, NC, USA). Jackson Immuno Research laboratory donkey anti-(sheep IgG) conjugated to horseradish peroxidase and rabbit anti-(goat IgG) conjugated to horseradish peroxidase was from Biocan Scientific (Mississauga, Ontario, Canada). Rabbit anti-(GST) antibody was from Santa-Cruz (California, USA).

2.2 Cell line

Human embryonic kidney (HEK 293) cells were employed for all tissue culture procedures throughout these studies. HEK 293 cells were transformed by exposing cells to sheared fragments of adenovirus type 5 DNA, creating the first adenovirus transformed human cell line (Graham et al., 1977).

2.3 Molecular biology

2.3.1 Vectors

2.3.1.1 pRBG4

The expression vector pRBG4 was constructed by cloning the 740-base-pair BalI/SacII restriction fragment of the human cytomegalovirus immediate early gene into the *Sac* I and *Bam* HI sites of the BluescriptpSK M13+, using a synthetic oligonucleotide adapter. A 137-base-pair *Hinc* II/*Kpn* I restriction fragment of pSV2CAT containing an SV40 polyadenylation sequence was then cloned into the corresponding unique sites of the Bluescript-CMV construct to create pRBG4 (Lee et al., 1991).

2.3.1.2 pcDNA3.1

pcDNA3.1 vectors (Invitrogen, Carlsbad, CA, USA) are 5.4 kb and are designed for high level constitutive expression in a variety of mammalian cells. pcDNA3.1 contains a bovine growth hormone (BGH) polyadenylation signal and transcription termination sequence to enhance mRNA stability and the ampicillin resistance gene for selection and maintenance in *E. coli*. Insertion into the pcDNA3.1 vector places cDNA under the control of the human cytomegalovirus early gene promoter.

2.3.1.3 pGEX

pGEX are bacterial vectors designed for inducible, high level intracellular expression of genes or gene fragments as fusions with *Schistosoma japonicum* glutathione-S-transferase. The GST gene fusion vector has a *tac* promoter for chemical induction of expression and an internal *lac* I^q gene for use in any *E. coli* host. The vectors also contain a PreScission (pGEX-6P-1) or factor Xa (pGEX-5X-3) protease recognition site for cleaving the desired protein from the fusion product.

2.3.2 Anion Exchanger expression constructs

2.3.2.1 AE1

2.3.2.1.1 pJRC9

Human AE1 cDNA (pHB3) (Lux et al., 1989) on an *Acc* I- *Hind* III fragment was cloned into the *Hind* III and *EcoR* I sites of expression vector pRBG4 (Lee et al., 1991) using an *Acc* I- *EcoR* I linker. The resulting human AE1 expression construct was called pJRC9 and contains 27 bases of 5'-untranslated sequence and the full coding sequence (Casey, Ding & Kopito, 1995).

2.3.2.1.2 WTAE1

Human AE1 cDNA (a generous gift of Drs. A.M. Garcia and H. Lodish) was inserted into the *Hind* III and *Bam* HI sites of pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) (Vince & Reithmeier, 1998).

2.3.2.1.3 NANN and AAAA AE1 mutants

The CAII binding site, L886AADD was replaced with nucleotide sequences encoding LNANN and LAAAA in the two mutants using the

Clontech Transformer Site-Directed Mutagenesis kit and oligonucleotide primers from ACGT Corp (Toronto, Canada) (Vince & Reithmeier, 2000).

2.3.2.2 AE2

Mouse AE2 cDNA (Alper et al., 1988) was cloned into the unique *EcoR* I site in pRBG4 and orientation confirmed by sequence analysis (Lee et al., 1991).

2.3.2.3 AE3

2.3.2.3.1 AE3c

cDNA clones for rat full-length AE3 and AE3c were kindly provided by Dr. Gary Shull (Kudrycki et al., 1990; Linn et al., 1992). The *Xba* I/*EcoR* I fragment of the AE3c cDNA was cloned into those sites in the expression vector pRBG4 (Lee et al., 1991) to yield construct pJRC31 (Sterling & Casey, 1999).

2.3.2.3.2 Full-length AE3

An expression construct for full-length AE3 was prepared by replacement of the 5′ end of the pJRC31 coding region. PCR was performed using the rat full-length AE3 cDNA clone pRBAE2-1 as template with forward primer A6257G07 (CGG AAT TCT CTA GAT GAG ACT CCT AAG TCT TCA GCC) and reverse primer A6257G09 (TCC GAT CCA GCT TCT TCT TC). The resulting PCR product was digested with *Xba* I and *Nar* I and cloned into pJRC31, digested with the same enzymes, to produce construct pJRC32 (Sterling & Casey, 1999).

2.3.2.3.3 AE3tr

A deletion construct encoding the common region found in full-length AE3 and AE3c (designated AE3tr) was designed to contain an additional ATG start codon at the 5′ end. The truncated construct, called pJRC34, was prepared by a deletion PCR strategy (Sarkar & Sommer, 1990). In the first round of PCR, pJRC31 was the template for PCR with forward primer A7723F07 (CTG AGG CCT TGG CCA CCA GGG GAA TGT GTC ATC GGC TGG AGG ACA ACC CG) and reverse primer A6257G09. This megaprimer was isolated and used as a reverse primer with forward primer A7728E03 (GCC ATC CAC GCT GTT TTG ACC), which has sequence similarity to the multiple cloning site of pRBG4. The resulting PCR product was digested with *Xba* I and *Nar* I and cloned into pJRC31 digested in the same way (Sterling & Casey, 1999).

2.3.2.3.4 pJRC10

An expression construct of glutathione S-transferase cDNA, fused to codons 7–69 of rat AE3c, was constructed by PCR with rat AE3c cDNA (from Dr. Gary Shull) as template, forward primer 2693 (CCG GGA TCC ACA AGG TCA TGG AGC CC) and reverse primer 2692 (CCG GGA TCC GCT CCC ACA GGC CAT AGT C), which introduced a *BamH* I site at each end of the coding sequence for the unique domain of AE3c. The PCR product was digested with *BamH* I and cloned into the *BamH* I site of pGEX-5X-3 (Amersham-Pharmacia Biotech) to yield pJRC10 (Sterling & Casey, 1999).

2.3.3 Carbonic anhydrase expression constructs

2.3.3.1 CAII

A construct for expression of wild-type human carbonic anhydrase II (CAII) was prepared from a CAII cDNA construct which was a kind gift from Dr. Carol Fierke (Fierke, Calderone & Krebs, 1991). The wild-type CAII was digested with *Xba* I at the 5′ end and *EcoR* I at the 3′ end. The *Xba*I-*EcoR* I fragment was cloned into *Xba* I and *EcoR* I digested pRBG4 vector (Lee et al., 1991), to yield pJRC36 (Sterling, Reithmeier & Casey, 2001).

2.3.3.2 V143Y mutant CAII

An expression construct for the mutant V143Y human CAII, called pDS14, was constructed from the V143Y cDNA construct which was generously supplied by Dr. Carol Fierke (Fierke et al., 1991). V143Y was digested with *Xba* I at the 5′ end and *EcoR* I at the 3′ end. The *Xba*I-*EcoR*I fragment was cloned into *Xba* I and *EcoR* I digested pRBG4 vector (Lee et al., 1991), to yield pDS14 (Sterling et al., 2001).

2.3.3.3 CAIV

An expression construct for the rabbit CAIV protein in pcDNA3 was received as a generous gift from Dr. George Schwartz (Schwartz et al., 2000).

2.3.4 DRA construct

An expression construct for human DRA in pCIneo was received as a generous gift from Dr. Manoocher Soleimani (Melvin et al., 1999).

2.3.5 GST-fusion protein expression constructs

2.3.5.1 GST-AE1ct

A GST-fusion protein consisting of the cDNA for glutathione-S-transferase fused to the 33 amino acid C-terminal tail of human AE1 (amino acids 879-911), was a generous gift from Dr. Reinhart Reithmeier and has been described previously (Vince & Reithmeier, 1998).

2.3.5.2 GST-DRAct

The GST-fusion protein consisting of the C-terminal 42 amino acids of human DRA was constructed using PCR to amplify the appropriate cDNA sequence. Using human DRA cDNA (Greeley et al., 2001) as template, the forward and reverse primers CGC GGA TCC AAG AAA GAT TAC AGT ACT TCA AAG TTT AAT CC and CGC GGA TCC GAA TTT TGT TTC AAC TGG CAC CTC ATA TAC CCA CT respectively, introduced a *BamH* 1 restriction site at both ends of the amplified product. The PCR product was digested with *BamH* 1 and ligated into the pGEX-6P-1 expression vector (Pharmacia Biotech), digested in the same way, to produce the construct GST-DRAct.

2.3.5.3 GST-EC3

A bacterial expression construct encoding a GST-fusion protein consisting of the cDNA for glutathione-S-transferase fused to the third (amino acids 560-584 - 5′-IFQDYPLQESYAPVVMKPKPQGPVP-3′) extracellular loop of rat AE1 (RSp19-7 (Kudrycki & Shull, 1993)) was constructed. Using rat AE1 as a template, the forward and reverse primers 5′-CGC GGA TCC TGA TTT TCC AGG ACT ACC CGC TAC-3′ and 5′-CGC GGA TCC TCA GGG CAC GGG GCC CTG AGG TTT-3′ respectively, introduced a *BamH* 1 site at both ends of the amplified third loop. The PCR product was digested with

BamH 1 and ligated into the pGEX-5X-3 expression vector (Pharmacia Biotech), digested in the same way, to produce the construct GST-EC3.

2.3.5.4 GST-EC4

A bacterial expression construct encoding a GST-fusion protein consisting of the cDNA for glutathione-S-transferase fused to the fourth (aa 643-677 - 5'TYTQKLSVPDGLKVSNSSARGWVIHPLGLYNHFPK-3') extracellular loop of AE1 was constructed. Using rat AE1 (RSp19-7 (Kudrycki & Shull, 1993)) as template the forward and reverse primers 5'-CGC GGA TCC TGA CCT ACA CGC AGA AAC TCT CG-3' and 5'-CGC GGA TCC TCA CTT GGG GAA ATG GTT ATA CAG-3' respectively, introduced a *BamH* 1 site at both ends of the amplified fourth loop. The PCR product was digested with *BamH* 1 and ligated into the pGEX-5X-3 expression vector (Pharmacia Biotech), digested in the same way, to produce the construct GST-EC4.

2.4 Preparation of Plasmid DNA

Plasmid DNA for transfections was prepared using Qiagen columns (Qiagen Inc., Mississauga, Canada). Constructs were verified by DNA sequencing, performed by the Core Facility in the Department of Biochemistry, University of Alberta, with either an Applied Biosystems 373A DNA sequencer or a Beckman Instruments CEQ2000 DNA sequencer. The AAAA and NANN mutations were confirmed using a Pharmacia Biotech T7 sequencing kit.

2.5 Purification of GST-fusion proteins

The GST-fusion constructs were transformed into *E. coli* BL21 and a single colony used to inoculate 50 ml LB media. Following overnight growth

at 37 °C with shaking, this culture was used to inoculate 1.2 liters LB media (5 ml/200 ml). The culture was grown at 37 °C with shaking until the A_{600} was 0.6-1.0. Isopropylthiogalactoside (1 mM final) was added and growth allowed to continue for 2-6 hours. The culture was then centrifuged at 10,000 x g, 10 min and bacterial pellets resuspended in 4 °C PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4), containing protease inhibitors (complete mini protease inhibitor cocktail, Roche Applied Science, Quebec, Canada). Suspended cells were disrupted by sonication (4 x 1 min, power level 9.5 with model W185 probe sonifier (Heat systems-Ultrasonics Inc., Plainview, N.Y.)) and inoculated with Triton X-100 to a final concentration of 1% (v/v) with slow stirring for 30 min. Following centrifugation (15,000 x g, 10 min) the supernatant was transferred to glutathione Sepharose 4B (50% slurry equilibrated with PBS, Pharmacia Biotech) and allowed to incubate at room temperature with gentle agitation for 1-2 hours. The sample was centrifuged (500 x g, 5 min) and the pellet washed three times with PBS. The fusion proteins were eluted using 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0.

2.6 Calcium phosphate transfection

HEK 293 cells were transiently transfected using the calcium phosphate method (Graham & Van Der Eb, 1973; Ruetz et al., 1993). 3.8 µg of the appropriate construct and 5.6 µg empty vector were diluted in 250 mM $CaCl_2$ to a final volume of 590 µl. If cells were being co-transfected and more than one construct was used then the amount of empty vector was reduced accordingly. The diluted cDNA was added in a dropwise manner to the

same volume of 2 X HEPES-buffered saline (275 mM NaCl, 30 mM $Na_2PO_4.7H_2O$, 55 mM HEPES, pH 7.0) and left at room temperature for 5 minutes until a precipitate had formed. Precipitate was then added dropwise to a dish of cells plated at 20-25 % confluency (316 µl to a 60 mm dish and 865 µl to a 100 mm dish) and cells were left for two days before use.

2.7 Expression of proteins in HEK 293 cells

HEK 293 cells (Graham et al., 1977) were grown at 37 °C in an air/CO_2 (19:1) environment in Dulbecco's modified Eagle's medium (DMEM) (Canadian Life Technologies Inc., Burlington, Ontario, Canada), supplemented with 5% (v/v) foetal bovine serum (Canadian Life Technologies Inc.) and 5% (v/v) calf serum (Canadian Life Technologies Inc.). Cells were transiently transfected using the calcium phosphate method (Ruetz et al., 1993) and used two days post-transfection.

2.8 SDS-PAGE

Samples for electrophoresis were prepared in 1 X SDS-PAGE sample buffer (10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 0.5% (w/v) bromophenol blue, 75 mM Tris, pH 6.8). Supplementation with protease inhibitors was performed in some cases as noted. Samples were stored on ice while being sheared through a 26 gauge needle (Becton–Dickinson). Prior to analysis, samples were heated to 65 °C for 5 min and insoluble material was sedimented by centrifugation at 16000 x g, 10 min in an IEC Micromax microcentrifuge. Samples were resolved by SDS/PAGE on 8% or 12.5% (w/v) acrylamide gels (Laemmli, 1970) using Bio-rad mini-protean II apparatus.

2.9 Immunoblot analysis

Proteins were transferred from polyacrylamide gels to PVDF membranes by electrophoresis for 12 h at 30 V in a cold room, in buffer composed of 20% (v/v) methanol, 25 mM Tris and 192 mM glycine using Bio-rad mini-protean II apparatus. PVDF membranes were blocked by incubation for 1 h in TBST-M (TBST (0.1% (v/v) Tween-20, 137 mM NaCl, 20 mM Tris, pH 7.5), containing 5% (w/v) non-fat dry milk) and then incubated overnight with 10 ml of TBST-M containing a primary antibody. After washing with TBST, blots were incubated for 1 h with 10 ml of TBST-M containing a secondary antibody conjugated to horseradish peroxidase. After washing with TBST, blots were visualised using ECL® reagent and Hyperfilm® or a Kodak Image Station 440CF.

2.10 Antibodies

1658 is a polyclonal antibody directed against AE1 and used at a 1:3000 dilution (Tang et al., 1998). AE2 is also detected using this antibody at the same concentration. AP3 is a polyclonal antibody directed against the common C-terminal region of Full-length AE3, AE3c and AE3tr (Kobayashi et al., 1994) and cAE3-1 is an antibody directed against the unique N-terminus of AE3c (Sterling & Casey, 1999). Both of these antibodies were used at a 1:3000 dilution. The goat anti-rabbit CAIV antibody, a generous gift from Dr. George Schwartz (Schwartz et al., 2000), was used at a 1:5000 dilution The Serotec sheep anti-human CAII antibody is used at a 1:10000 dilution.. Blots probed with 1658, AP3 and cAE3-1 were incubated with 1:3000 diluted donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham–Pharmacia Biotech, Quebec, Canada). CAIV blots were

incubated with 1:5000 diluted rabbit anti-goat IgG conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories) and CAII blots were incubated with 1:10000 diluted donkey anti-(sheep IgG) conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories).

2.11 Anion-exchange-activity assay

Transiently transfected HEK 293 cells were grown on poly-L-lysine-coated coverslips in 60 mm dishes. Two days post transfection, coverslips were rinsed in serum-free medium and incubated in 4 ml of serum-free medium containing 2 μM BCECF-AM (37 °C, 30 min). Coverslips were mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer's buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, pH 7.4) containing either 140 mM NaCl or 140 mM sodium gluconate. Both buffers were continuously bubbled with air/CO_2 (19:1). Fluorescence changes were monitored in a Photon Technologies International (London, Ontario, Canada) RCR fluorimeter at excitation wavelengths 440 and 502.5 nm and emission wavelength 528.7 nm. Fluorescence data were converted to pH_i by calibration using the nigericin/high potassium method (Thomas et al., 1979). The polyether ionophore, nigericin, couples the K^+ and H^+ gradients across the plasma membrane and when used in the presence of high K^+ (140 mM), will clamp pH_i at a known value. Incubating cells with nigericin/high potassium at pH values of 6.5, 7.0 and 7.5. calibrates fluorescence signals to provide an indirect measurement of changes in pH_i. Transport rates were determined by linear regression of the initial changes in

pH_i, using Kaleidagraph software (Synergy Software, Reading, PA, U.S.A.). All measurements of transport activity were corrected for background by subtraction of the transport activity of sham-transfected cells.

2.12 Inhibition of anion exchange transport activity

Transfected HEK 293 cells were grown on poly-L-lysine-coated coverslips in 60 mm dishes and subjected to the anion exchange transport assay described above. To assess the effect of the anion-exchange inhibitor 4,4′-di-isothiocyanostilbene-2,2′-disulphonate (DIDS), cells were subjected to a standard transport assay and then incubated with 100 μM DIDS in gluconate-Ringer's buffer for 10 min. Residual transport activity was then assessed after washing the cuvette with gluconate-Ringer's buffer for 300 s, followed by a standard transport assay (Tang et al., 1998). To ascertain the impact of the CAII inhibitor, acetazolamide, was perfused through the cuvette for 10 min after a standard transport assay. After washing with gluconate-Ringer's buffer, residual transport activity was monitored in a standard assay with acetazolamide present in all buffers (Sterling et al., 2001).

2.13 Measurement of intrinsic buffering capacity and proton flux

Intracellular buffering-capacity measurements were made by the ammonium-pulse method (Silva et al., 1997). HEK 293 cells grown on coverslips were sham-transfected and two days post transfection, cells were loaded with BCECF-AM as described above. Coverslips were mounted in a fluorescence cuvette and allowed to equilibrate in Ringer's buffer, without $NaHCO_3$. Cells were then perfused consecutively for 200 s with Ringer's buffer without $NaHCO_3$, containing 20, 10, 5, 1 and 0 mM NH_4Cl. $[NH4^+]_i$ was

calculated from the Henderson–Hasselbalch equation, and the intrinsic buffering capacity (β_i) was then calculated as $[NH_4^+]_i / pH_i$ (Silva et al., 1997). The total buffering capacity of the system (β_{total}) was then determined as $\beta_{total} = \beta_i + \beta_{CO2}$, where $\beta_{CO2} = 2.3[HCO_3^-]$ (Roos & Boron, 1981). Total proton flux was calculated as: $J_H^+ = \beta_{total} \times \Delta pH_I$.

2.14 Determination of perfusion interval

To determine the time taken for intracellular pH to equilibrate across the plasma membrane, we monitored pH_i in HEK 293 cells plated onto coverslips, transfected with AE1 cDNA and loaded with BCECF-AM as described above. Coverslips were mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer's buffer containing 5 µM nigericin, and either 140 mM KCl or 140 mM KNO_3. The pH of the Ringer's perfusion buffer was maintained at values 6.0, 6.5, 7.0 and 7.5. Fluorescence changes were monitored in a Photon Technologies International RCR fluorimeter at excitation wavelengths of 440 and 502.5 nm and an emission wavelength of 528.7 nm.

2.15 pH dependence assay

HEK 293 cells were plated onto coverslips and transfected as described above. Approximately 24 h post-transfection cells were incubated for 12-16 hours at 37 °C in 4 ml of serum-supplemented Dulbecco's modified Eagle's medium (see above), containing 10 mM SPQ . Coverslips were then mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer's buffer containing 5 µM nigericin, and either 140 mM KCl or 140 mM KNO_3. pH of the Ringer's perfusion buffer was maintained at values 6.0, 7.0, 8.0 and

9.0, thereby clamping pH_i at these values. At pH 6.0, HEPES in the Ringer's buffer was replaced with 10 mM Mes, and at pH 8.0 and 9.0, HEPES was replaced with 10 mM Tris. Fluorescence was monitored at excitation wavelength 350 nm and emission wavelength 438 nm in a Photon Technologies International RCR fluorimeter. To correct for changes of fluorescence that occurred due to dye leakage from cells and cell loss from the coverslip, total changes in fluorescence were normalised. The maximum fluorescence value observed in each transport cycle was set to 1.0 and the minimum was set to 0. Relative transport rates were determined by linear regression of the initial linear phase of each transport curve, using Kaleidagraph software. DIDS inhibition assays were performed as described above at $pH_o = pH_i = 6.0$ on cells expressing either full-length AE3 or AE3c and loaded with SPQ.

2.16 Cell surface processing

Transiently transfected HEK 293 cells were grown in 60 mm tissue culture dishes. Two days post transfection, cells were washed with PBS and harvested by incubation for 5 min at 37 °C with trypsin at 1 mg/ml in PBS. Cells were washed with 4 °C borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM $CaCl_2$, 10 mM boric acid, pH 9.0), and then incubated for 30 min at 4 °C in 3 ml of 0.5 mg/ml Sulpho-NHS-SS-Biotin in borate buffer. After washing three times with cold quenching buffer (192 mM glycine, 25 mM Tris, pH 8.3), cells were solubilised on ice in 500 µl of IP buffer (1% (w/v) deoxycholic acid, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 150 mM NaCl, 1 mM EDTA, 10 mM Tris/Cl, pH 7.5) containing protease inhibitors (0.1 mM PMSF, 0.2 mM tosyl-

phenylalanylchloromethane (TPCK), 0.1 mM tosyl-lysylchloromethane (TLCK)). The cell lysate was centrifuged for 20 min at 16000 x g in an IEC Micromax microcentrifuge and the supernatant collected. Half of the supernatant was retained for later SDS/PAGE analysis. Immobilised streptavidin resin (50 µl of streptavidin settled gel as a 50% (v/v) slurry in PBS containing 2 mM NaN_3) was added to the remaining supernatant, which was then incubated overnight at 4 °C with gentle rocking. Samples were centrifuged for 2 min at 8000 x g, and the supernatant collected and retained. Resin was washed five times with IP buffer and proteins were then eluted from the resin by addition of 250 µl SDS/PAGE sample buffer and incubation at 65 °C for 5 min. Three samples (total protein, unbound fraction and fraction eluted from resin) were analysed by SDS/PAGE and immunoblotted, as described above. Immunoblots were scanned with a Scanjet 4C flatbed scanner (Hewlett–Packard, Palo Alto, CA, U.S.A.), calibrated with a Q-14 grayscale (Kodak, Rochester, NY, U.S.A.). Scanned images were quantified using NIH Image 1.60 software (National Institutes of Health, Bethesda, MD, U.S.A.).

2.17 Quantification of CAII and AE1 expression

Human erythrocyte membranes were isolated by hypoosmotic lysis of erythrocytes (Casey, Lieberman & Reithmeier, 1989). Briefly, red blood cells, provided by the Canadian Blood Services, were washed 3 times in 0.9% NaCl, followed by centrifugation at 3000 x g, 5 minutes, in a JA-20 rotor. After each wash, the "buffy coat" of white cells present on top of the red cells, was removed by gentle aspiration. Red cells were osmotically lysed in ice-cold 5

mM sodium phosphate,.pH 8.0, supplemented with protease inhibitors (0.1 mM PMSF, 0.2 mM TPCK, 0.1 TLCK and 2 mM EDTA). Red cells were kept on ice for 10 minutes, before centrifugation, to allow lysis to occur. Membranes were sedimented by centrifugation at 27,000 x g, 20 minutes at 4 °C. The ghost membranes were washed by repeated suspension and centrifugation in ice-cold 5 mM sodium phosphate, pH 8.0, supplemented with protease inhibitors, until all heamoglobin was removed. A sample of the membranes was subjected to SDS-PAGE and Coomassie blue staining. The fraction of AE1 protein in the sample was assessed by densitometry. When combined with the concentration of protein in the sample (Bradford, 1976), this provided a measure of the concentration of AE1 in the sample. The stoichiometry of AE1 protein to CAII protein was determined by immunoblotting. HEK 293 cells were transiently transfected with either cDNA encoding for AE1 or with empty vector as described previously, the density of cells in each sample was determined by cell counting and cells were solubilised in SDS-PAGE sample buffer. Samples of the transfected cells were subjected to SDS-PAGE together with a range of amounts of erythrocyte membranes and known amounts of purified CAII protein standard (Vince & Reithmeier, 1998). Immunoblots were probed with anti-AE1 antibody 1658 and anti-CAII antibody (Serotec) and developed as described above, using a Kodak Image Station 440CF. The amount of AE1 and CAII in each cell sample was determined by densitometry of the immunoblots (Kodak 1D3.5 software) and comparison to the protein standards.

2.18 CAII Binding Assay

The binding of GST fusion proteins of the carboxyl-terminus of AE1 (GST-Ct) to CAII immobilised on microtiter plates was carried out as described previously (Vince & Reithmeier, 1998). Briefly, GST fusion proteins were expressed in *E. coli* BL21 and purified using glutathione Sepharose and DEAE-Sepharose (Vince & Reithmeier, 2000). Purified CAII or an inactive mutant (V143Y) (0.2-1 mg protein/well) were chemically coupled to 96 well microtiter plates (Corning Life Sciences) using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate. Plates were washed with antibody buffer (100 mM NaCl, 0.05% (v/v) Triton X-100, 5 mM EDTA, 0.25% (w/v) gelatin, 50 mM Tris-HCl, pH 7.5) and incubated with various concentrations (0-200 nM) of purified GST or GST-Ct in antibody buffer. Bound fusion proteins were detected by incubating the plates sequentially in goat anti-GST antibody, biotinylated affinity-purified anti-goat IgG, and peroxide-labeled biotin/avidin. This was followed by incubation with the peroxidase substrate o-phenyldiamine and detection of enzymatic activity at 450 nm in a Molecular Devices ThermoMax microplate reader. Binding curves were fitted with Kaleidagraph software (Synergy Software, Reading, U.S.A.).

2.19 Sucrose Density Ultracentrifugation

HEK 293 cells were transfected as described previously with cDNA encoding either AE1 or CAIV or co-transfected with both cDNAs. The method used to isolate detergent insoluble, glysosphingolipid enriched lipid rafts was a modified version of the Brown and Rose protocol (Brown & Rose, 1992). Two days post transfection cells were solubilised on ice in 2 ml

extraction buffer (140 mM NaCl, 1% (v/v) Triton X-100, 25 mM HEPES, pH 7.5, supplemented with protease inhibitors 0.1 mM PMSF, 0.2 mM TPCK, 0.1 TLCK and 2 mM EDTA.) for 10 minutes. Subsequently, samples were treated with 10 strokes in a Dounce homogeniser and then centrifuged at 1,000 x g for 5 min. Samples were made up to 4% sucrose by addition of an equal volume of 8% sucrose in extraction buffer without Triton X-100 and overlaid on 8 ml 5-30% continuous sucrose gradients in which sucrose had also been dissolved in extraction buffer without Triton X-100. Gradients were centrifuged in a Beckman SW41 rotor at 200,000 x g for 16-24 hours at 4 °C and then 1 ml fractions were removed. A sample of each fraction was prepared for SDS-PAGE analysis by addition of an equal volume of 2x SDS-PAGE sample buffer. Samples of each fraction were resolved by SDS-PAGE on 8 or 12.5% acrylamide gels. Immunoblots were prepared and protein detected as described above.

2.20 Gel overlay assay

HEK 293 cells grown in 60 mm culture dishes were transiently transfected individually with cDNA encoding human AE1, mouse AE2, rat AE3 or rabbit CAIV, as described above. Two days post-transfection cells expressing an AE protein were solubilised in SDS-PAGE sample buffer and cells expressing CAIV were solubilised in 200 µl 0.2% (w/v) SDS supplemented with protease inhibitors (0.1 mM PMSF, 0.2 mM tosyl-phenylalanylchloromethane (TPCK), 0.1 mM tosyl-lysylchloromethane (TLCK) and 2 mM EDTA.). Samples were stored on ice while being sheared through a 26 gauge needle (Becton–Dickinson). Prior to analysis, samples

were heated to 65 °C for 5 min and insoluble material was sedimented by centrifugation at 16,000 x g for 10 min in an IEC Micromax microcentrifuge. Immunoblots of lysates of cells transfected with AE cDNA were prepared as described above. Immunoblots were blocked for 3 hours with 10% TBST-M then incubated overnight in 1% TBST-M, containing 200 µl of the cell lysate prepared from CAIV-transfected cells. Immunoblots were then washed 4 x 15 minutes in TBST and then probed for CAIV as described previously.

2.21 GST pull-down assay

GST fusion proteins of the third and fourth extracellular loop of AE1 were used in a GST pull-down assay. Briefly, 0.2 pmol of either GST alone, GST-AE1EC3 or GST-AE1EC4 was bound to 25 µl glutathione Sepharose beads. Cell lysates of sham or CAIV- transfected cells were prepared by solubilisation of cells (60 mm dish of cells) in 200 µl 0.2% (w/v) SDS supplemented with protease inhibitors. Lysates were applied to the resin and incubated overnight at 4°C with rocking. Samples were centrifuged, 1000 x g for 5 min, and the supernatant removed. The resin was washed three times with PBS and samples eluted by heating at 75 °C for 10 minutes in SDS-PAGE sample buffer. Samples were resolved by SDS-PAGE on 12.5% polyacrylamide gels, transferred to a PVDF membrane and probed for CAIV as described above.

2.22 Statistical analysis

Values are expressed ± standard error of measurement. Statistical significance was determined using a Student's paired t-test with $p<0.05$ considered significant. Statistical analysis was performed using

Kaleidagraph software (Synergy Software, Reading, PA, U.S.A.) and Sigma Plot software (ISSC Inc.).

Chapter 3

Transport activity of AE3 chloride/bicarbonate anion-exchange proteins and their regulation by intracellular pH[2]

[2]**Portions of this chapter have been previously published and are reproduced with permission from:**
Sterling, D. and Casey, J.R. (1999) Regulation by Intracellular pH and Transport Activity of AE3 Cl^-/HCO_3^- Exchange Proteins. *Biochemical Journal*, **344** (1), 221-229 © The Biochemical Scoiety

Transport activity of AE3 chloride/bicarbonate anion-exchange proteins and their regulation by intracellular pH

3.1 Introduction

Anion-exchanger (AE) proteins facilitate the electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane of mammalian cells and thus contribute to regulation of intracellular pH (pH_i), [Cl^-] and cell volume. The erythroid band 3 protein, also called AE1, was the first anion exchanger identified (Cabantchik & Rothstein, 1974). cDNA libraries from a range of other tissues were probed with the AE1 gene, leading to the identification of other members of the AE family. The anion exchanger family is now comprised of three members, AE1, AE2 and AE3 (Alper et al., 1988; Kopito et al., 1989).

The AE3 isoform is of particular interest because it is found in the excitable tissues, brain (Kopito et al., 1989),heart (Linn et al., 1992) and retina (Kobayashi et al., 1994). Heart and retina co-express two different isoforms of AE3, that result from alternative promoter usage (Kobayashi et al., 1994; Linn et al., 1995; Linn et al., 1992). The 5′-untranslated and the 5′-coding sequences of the full-length mRNA are contained within exons 1–6 and the corresponding sequences of the cardiac isoform of AE3 (AE3c) mRNA are contained within intron 6 (Linn et al., 1992). Exons 7–23 encode sequences common to both full-length AE3 and AE3c (Linn et al., 1992). Human full-length AE3 is 1232 amino acids long and AE3c contains 1034 amino acids, including the unique 73-amino-acid domain at the N-terminus (Figure 3.1) (Yannoukakos et al., 1994). AE3c is the most abundant AE protein expressed in the heart (Linn et al., 1995), whereas

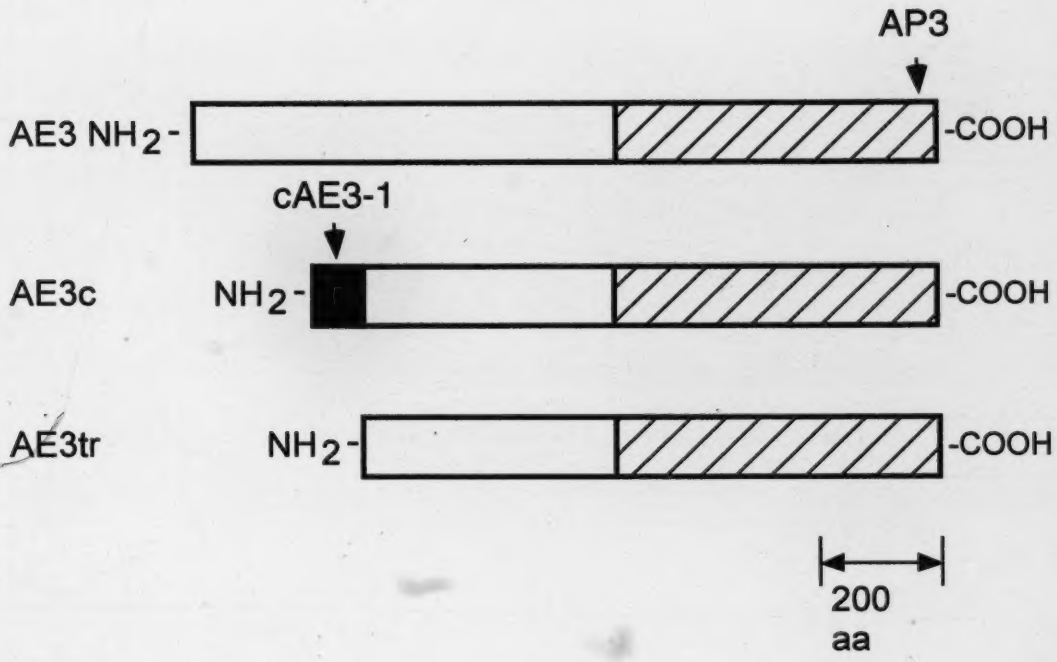


Figure 3.1 Structural homology of AE3 isoforms.

The C-terminal 957 amino acids of AE3 isoforms are identical, but the cardiac isoform has a unique 73 amino acid domain at its N-terminus (solid bar). AE3tr is an artificial construct that contains only the region in common between full-length AE3 and AE3c. The striped portions represent the transmembrane domain. Arrows indicate epitopes recognised by antibodies.

in the retina AE3c and full-length AE3 are expressed at similar levels, but in different retinal cell layers (Kobayashi et al., 1994).

pH_i modulates diverse cellular events, including ion-channel conductance (Ek-Vitorin et al., 1996), Ca^{2+} homeostasis (Busa & Nuccitelli, 1984), gene expression (Isfort et al., 1993) and cell death (Reynolds et al., 1996). In cardiac muscle, Ca^{2+} homeostasis and Ca^{2+} sensitivity of myofilaments, which closely regulate contraction, are affected by changes of pH_i (Orchard & Kentish, 1990). pH_i also modulates gap-junction conductance and, in turn, cardiac-cell electrical coupling (Ek-Vitorin et al., 1996; Hermans et al., 1995).

During a cardiac ischaemic episode, heart cells develop an acid load from which they must recover. Intracellular buffers and ion-transport proteins present in the plasma membrane mediate regulation of pH_i in cardiac cells. An understanding of the function and regulation of pH-regulatory proteins would help to elucidate their role in the recovery of pH_i in cardiomyocytes. Membrane-ion-transport proteins present in cardiomyocytes include the Na^+/H^+ antiporter (Sardet et al., 1989), the Na^+/HCO_3^- co-transporter (Romero et al., 1997), the lactate/H^+ co-transport proteins (Garcia et al., 1995) and isoforms of the AE family (Linn et al., 1995; Pucéat & Vassort, 1995). In addition to the two AE3 variants, cardiac cells also express AE2, at least at the mRNA level (Kudrycki et al., 1990). Full-length AE1 has been detected in the heart (Kudrycki et al., 1990), and a truncated form of AE1 has also been reported (Pucéat & Vassort, 1995).

The anion-exchange activity of AE1 and AE2, and the pH_i-sensitivity of transport have been studied previously (Humphreys et al., 1995; Jiang et al., 1994; Passow, 1986). AE1 transport activity is known to be relatively insensitive

to changes in pH_i over a physiological range (Passow, 1986). AE2, however, is under steep negative regulation at acid pH_i, consistent with its role in recovery from alkaline loading (Humphreys et al., 1995; Jiang et al., 1994). The regulation of full-length AE3 by pH_i has been reported previously (Lee et al., 1991), but regulation of AE3c has not been studied.

In the present study we used transiently transfected HEK 293 cells to examine the anion-transport activity of both the full-length and cardiac isoforms of AE3 that are found in the heart. We then determined the role of pH in regulation of AE activity. We found that full-length AE3 and AE3c are not sharply regulated by pH. The transport activity of both AE3 isoforms at acid pH suggests that these proteins may be involved in recovery from acid loading, by inward transport of HCO_3^-.

3.2 Results

3.2.1 Transient expression of AE cDNAs

All cDNA clones of interest for this study were inserted in the expression vector pRBG4, which places the insert under the control of the human cytomegalovirus immediate early gene promoter. The HEK 293 cell line was used in these experiments because these cells have very low anion-exchange-transport activity (Kopito et al., 1989). Immunoblots were probed with either AP3 (Kobayashi et al., 1994), a polyclonal antibody directed against the common C-terminus of all AE3 variants, or cAE3-1, a polyclonal antibody directed against the unique region of AE3c (Figure 3.1). Figure 3.2(A) shows that the three AE3 proteins are expressed to a very similar level. Cells transfected with pRBG4

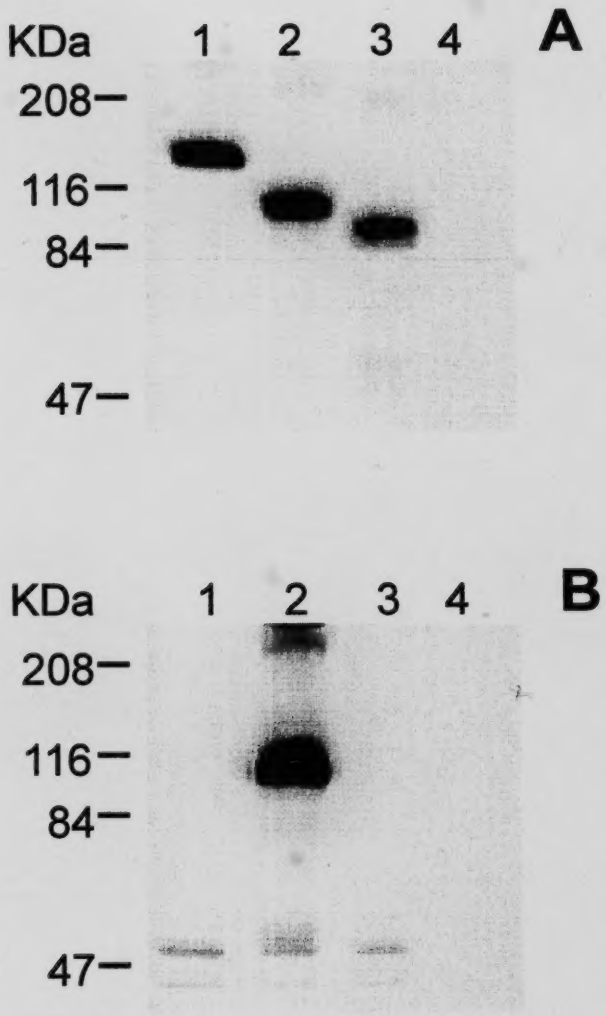


Figure 3.2 Immunoblots of AE3 isoforms.

Cell lysates were prepared from HEK 293 cells transfected with cDNA encoding: lane 1, full-length AE3; lane 2, AE3c; lane 3, AE3tr and lane 4, pRBG4 vector only. (A) Immunoblot probed with a 1:3000 dilution of the polyclonal anti-(AE3 C-terminus) antibody, AP3. (B) Immunoblot probed with a 1:5000 dilution of polyclonal antibody cAE3-1, directed against the unique N-terminus of AE3c.

vector alone had no immunoreactive material (Figure 3.2A). Figure 3.2(B) illustrates that the polyclonal antibody cAE3-1 detects AE3c only.

3.2.2 AE activity

The anion-exchange assay measures the rate of exchange of Cl^- for HCO_3^- across the plasma membrane mediated by the AE (Lindsey et al., 1990b). In this assay, transfected cells were grown on glass coverslips, loaded with BCECF-AM, a pH-sensitive fluorescent dye, and mounted in a fluorescence cuvette. The light beam in the fluorimeter illuminates approximately $1x10^4$ cells, so that fluorescence data represent the mean response of a large population of cells. After appropriate calibration, changes of fluorescence indicate changes in pH_i. In Cl^--free medium, Cl^- leaves the cell in exchange for HCO_3^- entry, which induces cell alkalinisation (Figure 3.3) (Lindsey et al., 1990b). Figure 3.3 shows typical anion-exchange data for AE3 isoforms. In these experiments, transport rates were determined from the initial slopes of the curves produced as alkalinisation occurs. To convert flux in units of change of pH/time to flux of proton equivalents/time, it was essential to determine the intrinsic buffering capacity of transfected HEK 293 cells. Figure 3.4 shows that the intrinsic buffering capacity of transfected HEK 293 cells was negligible at pH values above 7.2. The flux of proton equivalents in units of mM/min was therefore determined from the product of the change of pH/time and ($2.3 \times [HCO_3^-]$).

In anion-exchange assays carbon dioxide diffuses into cells to provide a source of bicarbonate for efflux. HCO_3^- is usually produced by CO_2 hydration, mediated by CA. To determine the importance of CA to the anion-exchange process, we used immunoblots to determine that CAII is endogenously

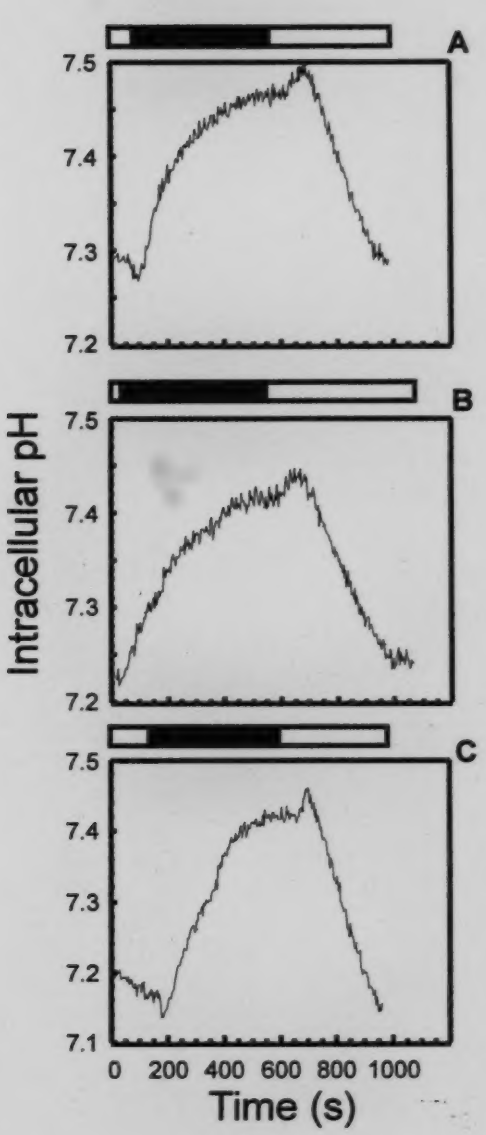


Figure 3.3 Anion-exchange-activity assay.

Transiently transfected cells, grown on coverslips, were loaded with BCECF-AM and perfused with Ringer's buffer containing either 140 mM NaCl (open bar) or 140 mM Na gluconate (solid bar) as indicated by the bar at the top of the figure. Excitation wavelengths were 440 nm and 502.5 nm and the emission wavelength was 528.7 nm. pH_i was monitored as outlined in the experimental section. Cells were transfected with cDNA encoding: (A), full-length AE3; (B), AE3c and (C), AE3tr.

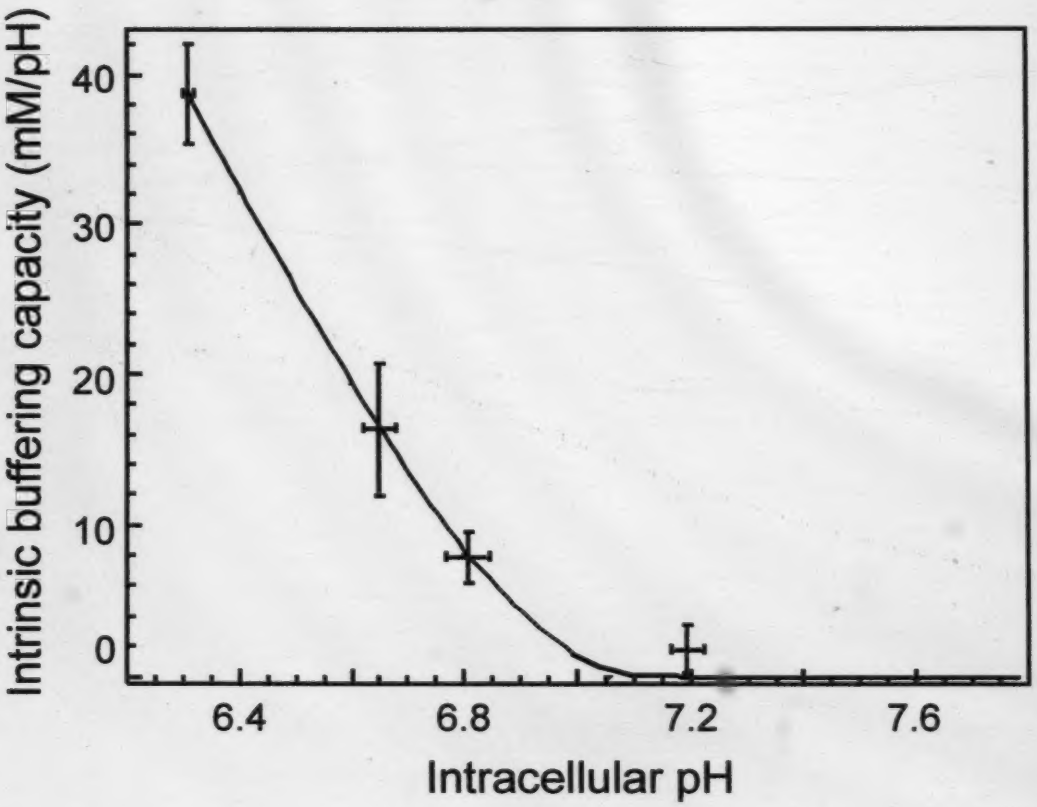


Figure 3.4 Intracellular buffering capacity of sham-transfected HEK 293 cells. Each point represents the mean of four measurements ± S.E.M.

expressed in HEK 293 cells (results not shown). The presence of CAII is essential to observe full anion-exchange activity since treatment of AE1-transfected cells with 100 μM acetazolamide resulted in nearly complete inhibition of anion transport activity (results not shown). To determine whether the endogenous level of CAII found in HEK 293 cells was sufficient to observe full anion-exchange activity, we co-transfected HEK 293 cells with expression constructs for AE1 and CAII. On the basis of immunoblots, CAII was expressed at levels many times the endogenous level. However, within experimental error the transport activity of AE1–CAII-co-transfected cells could not be distinguished from AE1-transfected cells (results not shown). Therefore co-transfection with CAII was not required to observe full anion-exchange activity; in subsequent experiments cells were not co-transfected with CAII.

Table 3.1 summarises the transport activity of AE isoforms. AE1 and AE2 had similar transport activities. This rate is approx. 8-fold higher than that of the AE3 isoforms, a finding that has been noted previously in oocytes (Humphreys et al., 1994; Yannoukakos et al., 1994). Negative-control cells transfected with pRBG4 vector alone displayed a level of 0.81 ± 0.11 mM H^+/min, which is a background rate of 2.5–22% depending on the AE isoform. The background activity of pRBG4-transfected cells was subtracted from all transport data presented here. All AE isoforms were sensitive to DIDS although to different extents. Following incubation with DIDS, the isoforms had the following residual activities: AE1, 18 ± 7%; AE2, 59 ± 7%; full-length AE3, 38 ± 1%; AE3c, 36 ± 6% (n = 3). Therefore AE2 and AE3 are much less sensitive to inhibition by DIDS than AE1.

Table 3.1 Anion-exchange activity and cell-surface processing.

Anion-exchange rates are shown as the mean ± S.E.M. (n=4) and have been corrected for background activity of cells transfected with pRBG4 vector alone. Cell-surface processing is expressed as a percentage of total AE protein present in the cell, and the S.E.M. is shown (n= 3).

AE isoform	Proton flux (mM/min)	Cell surface expression (%)
AE1	40 ± 1	31 ± 1
AE2	32 ± 3	32 ± 2
AE3	4 ± 1	30 ± 2
AE3c	4 ± 1	31 ± 1
AE3tr	4 ± 1	39 ± 1

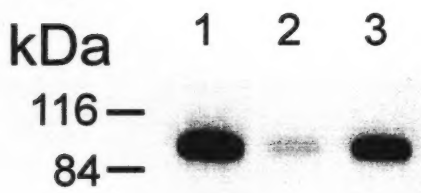


Figure 3.5 Cell-surface processing of AE1.

HEK 293 cells transfected with AE1 cDNA were labeled with Sulpho-NHS-SS-Biotin. Cells were solubilised in SDS/PAGE sample buffer. Half of the sample was loaded into lane 1 (total protein). The remainder was incubated with streptavidin–agarose, to remove biotinylated proteins. The unbound, supernatant fraction was loaded in lane 3. The beads were incubated at 65 °C to cleave the biotin–agarose linkage. The supernatant from this preparation was loaded in lane 2.

3.2.3 Surface processing

The range of transport activities for the AE1, AE2 and AE3 isoforms is striking (Table 3.1). Because anion-exchange assays only measure the functional activity of protein in the plasma membrane, differences in the apparent transport activity of the AE proteins could result from altered levels of processing to the cell surface. To examine this possibility, we measured the level of cell-surface expression of AEs in cells that had been transfected for the same time period as cells used for transport experiments. Membrane-impermeant Sulpho-NHS-SS-Biotin reacts only with primary amines of proteins in the plasma membrane. We identified the biotinylated cell-surface proteins by their binding to streptavidin resin. Figure 3.5 shows an example of cell-surface-processing results. The amount of AE1 present in lane 3 (fraction not biotinylated) is similar to the total amount of AE1 present (lane 1). Quantification of the data by densitometry showed that 69% of the protein was retained in an inaccessible intracellular location since it could not be biotinylated. The amount of AE1 present in the biotinylated fraction (lane 2) was much lower than the 31% estimated from the fraction that was labeled. We attribute this to an inability to elute bound AE1 from the resin. Table 3.1 shows that approximately 30% of total protein present of each isoform of AE is processed to the surface membrane. Therefore differences in cell surface processing do not explain the differences in anion-exchange activity that we observed. Similar amounts of AE protein were noted at the plasma membrane of AE-transfected cells using immunoflouresence (Jocelyne Fujinaga, unpublished observation).

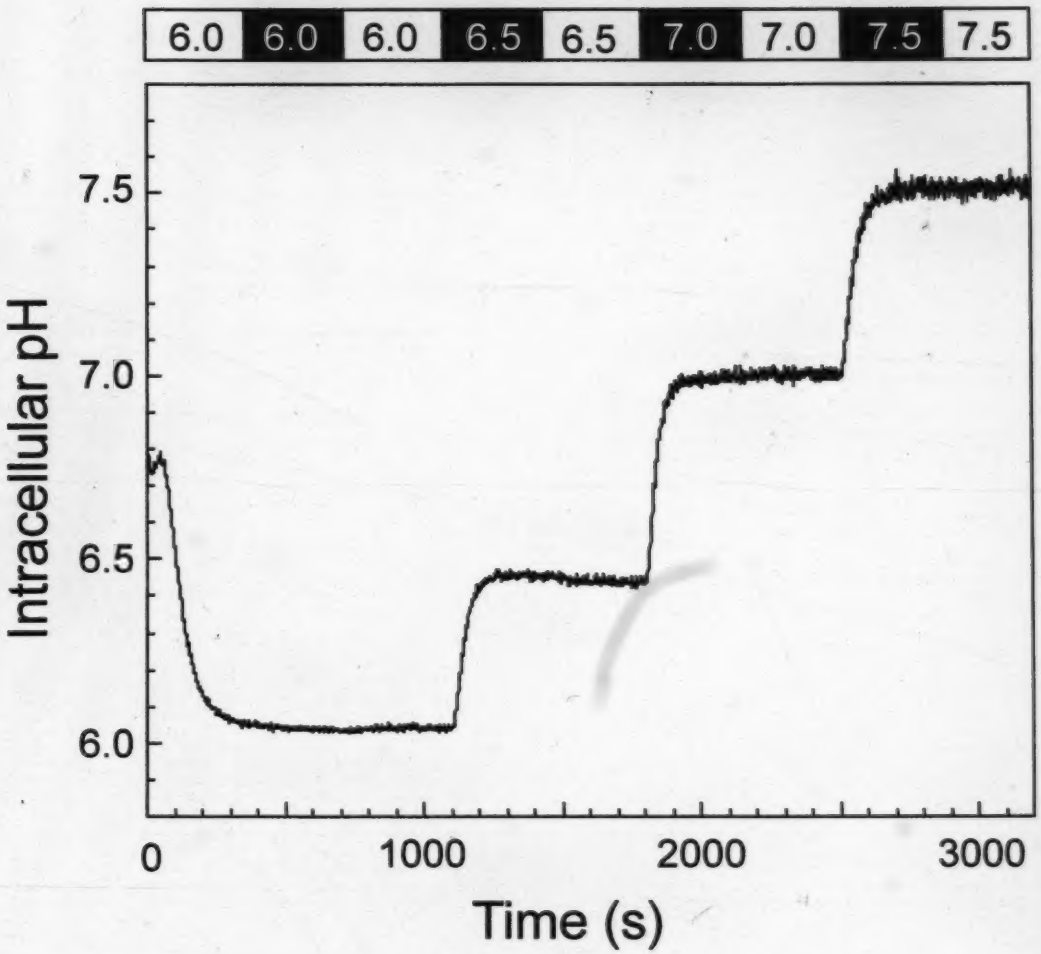


Figure 3.6 Perfusion interval required to change pH_i.

HEK 293 cells were grown on glass coverslips and transfected with AE1 cDNA. Two days post-transfection, cells were loaded with BCECF-AM and suspended in a fluorescence cuvette. The cuvette was perfused with high potassium Ringer's buffer at various pH values (see bar at the top of the figure), containing 5 μM nigericin and either 140 mM KCl (open bar) or 140 mM KNO_3 (solid bar), as indicated by the bar at the top of the figure. Fluorescence was measured with excitation wavelengths of 440 nm and 502.5 nm and an emission wavelength of 528.7 nm.

3.2.4 Determination of perfusion interval

Prior to measuring anion-exchange activity at various pH values, it was essential to determine the incubation period required to equilibrate pH_i. In this assay, we used nigericin, a K^+/H^+ ionophore, in the presence of high potassium to equilibrate pH across the plasma membrane (Thomas et al., 1979). This enabled us to alter pH_i by changing extracellular pH (pH_o). To determine the length of time required for pH_i to equilibrate with pH_o, we continuously monitored pH_i in BCECF-AM-loaded cells, while we changed the pH of the extracellular Ringer's medium. Figure 3.6 shows that pH_i rapidly changed upon change of pH_o and pH_i reached equilibrium within 200 s. Figure 3.6 also shows that change of medium from chloride-Ringer's to nitrate-Ringer's had no effect on pH_i. The results presented in Figure 3.6 cover only the pH 6–7.5 region, because the BCECF-AM dye (pKa = 6.8) did not accurately report pH_i beyond this pH range. The pH dependence of BCECF-AM did not affect experiments designed to monitor transport activity in the pH 6–9 range (see below) because SPQ dye was used to follow chloride fluxes.

3.2.5 pH-dependence assay

To follow anion transport at various pH values, we loaded transfected cells with SPQ. Fluorescence of this chloride sensitive dye becomes quenched in the presence of chloride, but not nitrate (Illsley & Verkman, 1987). We therefore monitored changes of intracellular [Cl^-], as chloride exchanges for nitrate, a substrate of the AE. In these assays, cells were alternately perfused with chloride- or nitrate-containing Ringer's buffer. As pH levels can only drop as low as 6.2 during an ischaemic episode (Orchard & Kentish, 1990), assays were

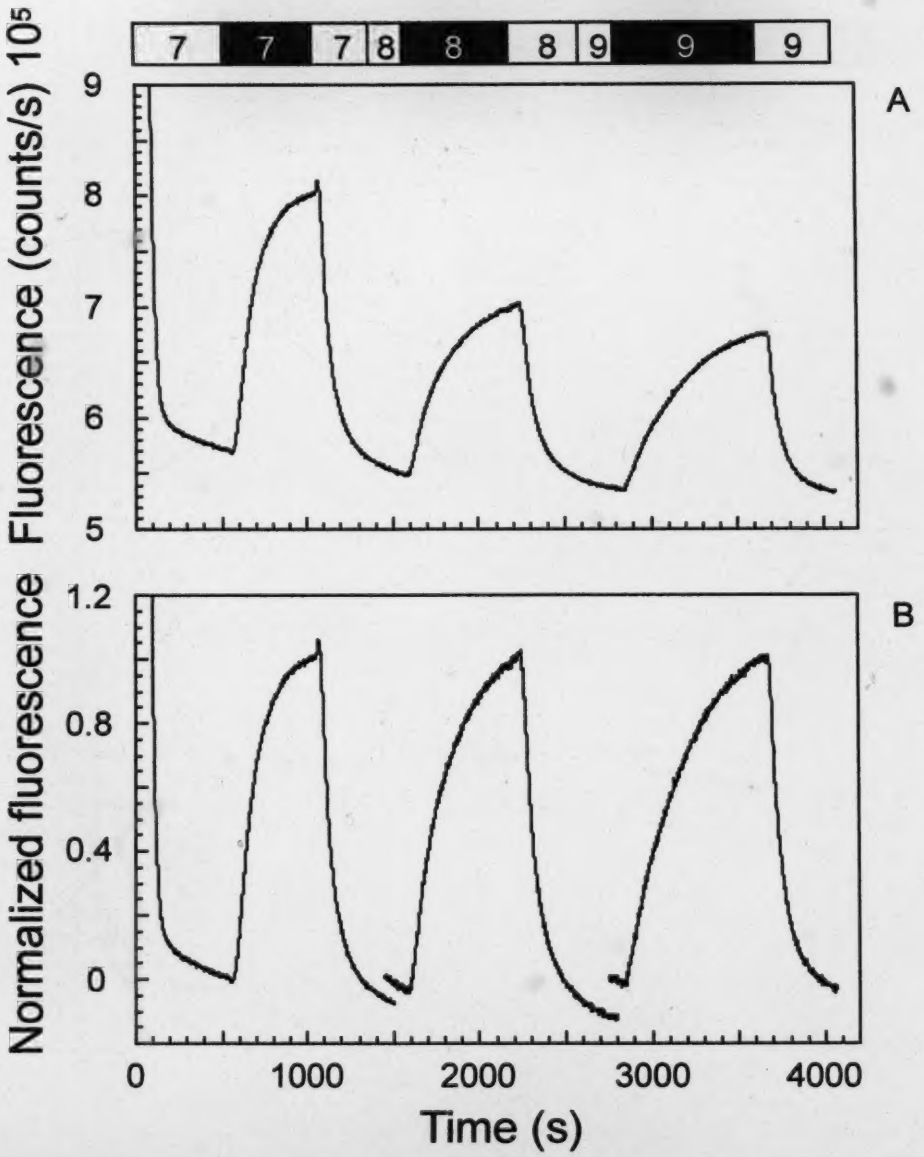


Figure 3.7 Measurement of anion exchange at various pH values.

HEK 293 cells, grown on coverslips, were transiently transfected with full-length AE3 cDNA. Cells were loaded with SPQ dye and suspended in a fluorescence cuvette. Cells were perfused with Ringer's buffer at various pH values (see bar at the top of the figure), containing 5 μM nigericin and either 140 mM KCl (open bar) or KNO_3 (solid bar), as indicated by the bar at the top of the figure. Fluorescence was measured with an excitation wavelength of 350 nm and an emission wavelength of 428 nm. Raw transport data (A) were normalised to the same total change in fluorescence in each transport curve (B).

performed at pH values of 6.0, 7.0, 8.0 and 9.0 to give a full range of AE activity versus pH.

Figure 3.7 shows data from a representative anion transport experiment, with varied pH_o = pH_i. The figure shows AE3-transfected cells undergoing perfusion with chloride- and nitrate-containing Ringer's buffer at pH 7.0, 8.0 and 9.0. The maximum unquenched fluorescence level always decreased after the first curve regardless of the pH values used in the experiment. We attribute the decrease in fluorescence to loss of cells from the coverslip and SPQ leakage from cells. To correct for cell and dye loss, each pH transient was normalised to the same fluorescence range. The correction method was validated by experiments in which transport was measured three consecutive times on the same cell sample, at pH 7.0. After normalisation of the fluorescence range, measured transport activity was consistent for all three determinations.

In these experiments, transport rates were determined from the initial slopes of the chloride influx or efflux curves. To ensure that changes of pH_i did not cause irreversible changes to the cell, we monitored transport activity in cells exposed to the pH sequences (6.0, 7.0), (7.0,6.0,), (7.0, 8.0, 9.0) and (9.0, 8.0, 7.0). Each coverslip was exposed to only one sequence of pH values. In each case pH 7.0 was included in the series to allow for normalisation between experiments.

Figure 3.8 summarises regulation by pH of each AE isoform. AE1 and AE2 served as controls representing AEs that are known to be relatively pH-insensitive (Passow, 1986) and pH-sensitive (Jiang et al., 1994; Lee et al., 1991), respectively. Our results are consistent with previous determinations of the pH-dependence of AE1 and AE2 transport activity. Our results show that the anion

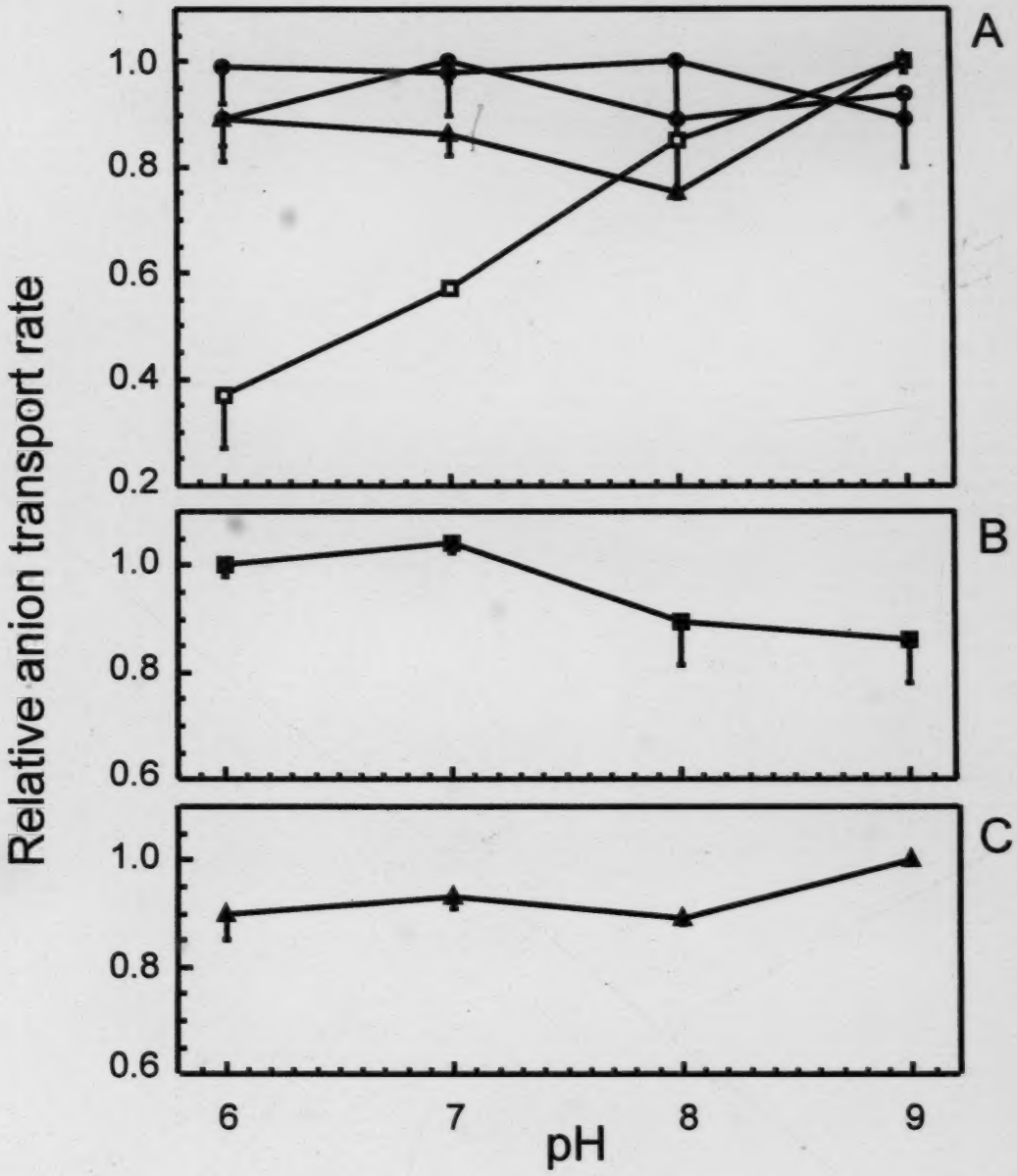


Figure 3.8 Regulation of anion exchange by pH_i.

Anion exchange rates were measured at various pH_i values. Transport rates are plotted relative to the maximum rate observed for each isoform and error bars represent the S.E.M. (n = 8). (A) Open circles, AE1; open squares, AE2; open triangles, full-length AE3; closed circles, AE3c. AE3tr activity is shown in (B) and pRBG4 activity in (C).

transport activities of full-length AE3, AE3c and AE3tr are relatively pH independent. Figure 3.8 shows that background chloride flux in cells transfected with pRBG4 vector alone did not vary with pH. The absolute level of the background transport activity could not be assessed in Cl^-/NO_3^- exchange assays, but represented only 2.5–22% of total transport when measured in Cl^-/HCO_3^- exchange assays (Table 3.1). Cells expressing either full-length AE3 or AE3c were similarly sensitive to DIDS when pH_i is 6.0 as at physiological pH_i. Anion transport at pH 6.0 can therefore be attributed to the AE and not to other processes that may come into effect at lower pH_i values.

3.3 Discussion

In the present study, we examined the transport activity of AE1, AE2 and AE3 isoforms and their regulation by pH. AE1- and AE2-transfected cells had the highest anion-exchange activity (40 and 32 $mM \cdot min^{-1}$, respectively). The AE3 isoforms have approximately one eighth the activity of the AE1- and AE2-transfected cells. All of the AE3 isoforms had anion transport activity that was not inhibited by acid pH_i. This is the first paper to assess the transport activity and regulation by pH of AE1, AE2 and AE3 isoforms in one study. AE3c and the AE3tr mutant have not previously been heterologously expressed in mammalian cells and functionally characterised.

Two previous papers have reported the activity of full-length AE3, after expression in HEK 293 cells (Lee et al., 1991) and in X. *laevis* oocytes (Yannoukakos et al., 1994) and found different results. Our results are consistent with a report that in HEK 293 cells full-length AE3 activity was 8-fold lower than that of AE2 (Yannoukakos et al., 1994). Both full-length AE3 and AE3c had

similar levels of transport activity, approx. 2.5 times that in water-injected cells, when expressed in X. *laevis* oocytes (Yannoukakos et al., 1994). By contrast oocytes injected with cRNA for AE1 and AE2 had ion fluxes that were > 10–20 - fold above those in water-injected cells (Humphreys et al., 1994). Thus in X. *laevis* oocytes, AE3 isoforms are about 8-fold less active than AE1 or AE2.

We reasoned that the degree of cell-surface processing of the AE proteins could explain the large differences in their transport activity. Our finding that a similar fraction (approx. 30%) of each AE isoform is processed to the plasma membrane does not explain the differences in transport rates. However, because we do not have an antibody that detects both AE2 and AE3, we cannot rule out the possibility that the transport rates differ because of differences in the level of protein expression. Since the membrane domains, which carry out anion exchange, are 90% identical between AE2 and AE3, the more divergent cytoplasmic domains (53% identity) may account for the differences in transport activity. The cytoplasmic domains of AE proteins may interact with the membrane domain to regulate activity. Indeed, the transport studies of chimeric proteins of AE1 and AE2 membrane and cytoplasmic domains showed that both domains contribute to regulation of anion transport by pH (Zhang et al., 1996).

Alternatively, tissue-specific cofactors may be required for full function of the AE3 isoforms. In contrast with the HEK 293 cells used in the present study, excitatory cells (heart and retina), where AE3 isoforms are found, may provide the optimal environment for AE3 transport regulation.

Indirect evidence suggests that AE function is responsive to kinases (Baggio et al., 1993; Jiang et al., 1994; Olsnes, Tonessen & Sandvig, 1986). Anion-

exchange activity in the heart is stimulated by β-adrenergic agonists (Désilets et al., 1994), purinergic agonists (Pucéat et al., 1991) and angiotensin II (Alvarez et al., 2001; Grace & Vandenberg, 1993). A role for tyrosine kinases is suggested by the observation that purinergic activation of anion-exchange activity in cardiomyocytes is inhibited by tyrosine kinase inhibitors (Puceat et al., 1998). Tyrosine-kinase-coupled receptors transmit some of their signal through SH3 proteins (Pawson, 1994; Pawson & Gish, 1992). Interestingly, the cytoplasmic domain of full-length AE3 contains two potential SH3-binding sites, one of which is flanked by 11 consecutive glutamic acid residues, giving it the appearance of a charged-protein-binding site.

In the heart, AE3c is expressed at a much higher level than full-length AE3 (Linn et al., 1995). It is possible that the unique domain of AE3c serves as a scaffolding site for either peripheral or cytoskeletal proteins. In this case, the reduced activity of AE3c limits the total anion transport capacity of the cell, while increasing the number of plasma membrane binding sites. Precedent for AEs as plasma membrane attachment sites comes from the erythrocyte AE1 protein, whose cytoplasmic domain anchors the plasma membrane to the cytoskeleton through interaction with ankyrin (Low, 1986).

The range of transport rates of AE isoforms (Table 3.1) has significance in those tissues, such as the heart, that express multiple AE isoforms. Northern blots show that cardiac tissue expresses AE1, AE2, full-length AE3 and AE3c (Kudrycki et al., 1990). Northern blots of rat heart mRNA indicate that the expression level of AE isoforms is: AE3c >> full-length AE3 > AE2 > AE1 (results not shown). However, since each isoform was probed with a different cDNA,

the quantification is not completely reliable. Since the anion-exchange activities of AE1 and AE2 are much greater than the AE3 isoforms, AE1 and AE2 may have a more significant role in the heart than is suggested by consideration of their mRNA expression levels only.

Results presented here demonstrate differential regulation of AEs by pH. In the present study we have confirmed that AE1 transport activity is relatively insensitive to changes in pH (Passow, 1986) and that AE2 transport activity is steeply negatively regulated by acid pH_i, consistent with its role in cellular acidification (Humphreys et al., 1994; Jiang et al., 1994). Previous researchers reported that when expressed in CHOP cells, AE2 was only 20% active at pH 7.3 and had approx. 8% of full activity at pH 6.9 (Jiang et al., 1994). In the present study we found that AE2 was 65% active at pH 7.3 and activity was reduced to 37% of maximum at pH 6.0. Half-maximal activity was attained at pH 7.4, which is close to the value of pH 7.5 found in CHOP cells. Therefore the pH of half-maximal activity is consistent between the two studies, but we found that AE2 was less sensitive to acid pH than the previous group. One possible explanation for the result is that in the present study both pH_i and pH_o were changed together, while in the CHOP cell study pH_o was held constant and pH_i was varied using pulses of NH_4Cl.

In contrast with our results, one previous study found that the anion-exchange activity of full-length AE3 is inhibited by acid pH_i (Lee et al., 1991). In that study, Cl^-/HCO_3^- exchange was quantified by the rate of change of pH_i, measured by following the rate of pH_i recovery in cells made acidic or alkaline. Thus the variable under study (the effect of pH_i) varied constantly during

measurement of transport. By contrast, in the present study pH_i was clamped at a constant value during each transport assay. Therefore pH_i did not change during our measurement of transport activity (Figure 3.6), which allowed for better measurement of initial transport rates at different pH_i values. Alternatively, in the previous experiment only pH_i was varied, while in our experiments pH_i and pH_o were both simultaneously varied. We cannot rule out the possibility that AE3 transport is positively regulated by acid pH_o and negatively regulated by acid pH_i. The results of this study clearly show that transport by AE3 isoforms is essentially independent of changes in pH_o and pH_i. Although AE3c was previously shown in *X. laevis* oocytes to be a functional AE (Yannoukakos et al., 1994), the present study is the first examination of pH regulation of AE3c and AE3tr.

The retina is the only other tissue found to express both full-length AE3 and AE3c (Kobayashi et al., 1994). Interestingly, heart and retina both contain an extensive network of electrically coupled cells whose gap-junctional conductances are extremely sensitive to changes in pH_i. A 0.2–0.3 unit decrease in pH_i will lead to a 50% decrease in junctional conductance in the retina (DeVries & Schwartz, 1989), and a decrease of pH_i of 0.3 will cause a 30–80% decrease in junctional conductance in the heart, with gap junctions composed of connexin 45 being the most affected (Hermans et al., 1995). It is clear that maintenance of alkaline pH_i is of extreme importance in both retina and heart. We therefore propose that AE3 isoforms in the retina and heart are present to alkalinise these cells by inward movement of bicarbonate. Our observation of AE3 activity under acid conditions underscores this possibility.

The central role of the CO_2/HCO_3^- buffer system for pH homeostasis is dependent on CA, an enzyme that catalyses the hydration–dehydration of CO_2/HCO_3^- (Maren, 1967). The CA isoform present in cardiomyocytes is CAIV (Vandenberg et al., 1996), which is linked extracellularly by a glycosylphosphatidyl inositol anchor to the plasma membrane (Waheed et al., 1992). CAIV has a significant role in facilitating the recovery of pH_i after reperfusion of ischaemic heart (Vandenberg et al., 1996). We attribute this to the movement of freely diffusible CO_2 out of the cell and its rapid conversion to HCO_3^- by CAIV. The extracellular HCO_3^- could then be exchanged for intracellular Cl^- by AE3 proteins, leading to cellular alkalinisation.

In the present study we have investigated the transport activity of both full-length AE3 and AE3c and compared them to that of AE1 and AE2. Our results indicate that although a similar fraction of each AE is processed to the plasma membrane, the rate of anion transport by the AE3 isoforms is much lower than that of both AE1 and AE2. Our finding that anion transport by AE3 isoforms is essentially independent of changes in pH_o/pH_i indicates that these proteins may contribute to pH_i recovery following an acid load. Further studies are needed to determine which regulatory processes are important in the control of anion exchange by AE3 isoforms.

Chapter 4

A Transport Metabolon: Functional Interaction of Carbonic Anhydrase II and Chloride/Bicarbonate Exchangers[3]

[3]**Portions of this chapter have been previously published:**
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Sterling, D., Reithmeier, R.A.F. and Casey, J.R. (2001) Carbonic Anhydrase: In the Driver's Seat for Bicarbonate Transport. J. Pancreas **2** (4), 165-170

A Transport Metabolon: Functional Interaction of CAII and Chloride/Bicarbonate Exchangers

4.1 Introduction

Carbon dioxide, the metabolic end-product of oxidative respiration, must be effectively cleared from the human body. CO_2 diffuses out of cells, into the blood stream and into erythrocytes where it is hydrated by cytosolic carbonic anhydrase (CA). The resulting membrane-impermeant HCO_3^- is exported into the plasma by the plasma membrane Cl^-/HCO_3^- anion exchanger (AE1), thus increasing the blood's capacity for carrying CO_2. Upon returning to the lungs the process is reversed; HCO_3^- is transported into the erythrocyte in exchange for Cl^- by AE1, dehydrated by CA, and the resulting CO_2 diffuses across the erythrocyte and alveolar membranes to be expired from the body. The 5×10^4 s^{-1} turnover rate of AE1 (Jennings, 1989) and the high content of AE1 in the membrane (Steck, Fairbanks & Wallach, 1971) facilitate completion of bicarbonate transport within 50 ms during passage of an erythrocyte through a capillary (Lodish et al., 2000).

AE1 is a 911 amino acid polytopic glycoprotein, which facilitates the one for one electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane. It consists of two major domains, a 43 kDa amino-terminal cytoplasmic domain (Steck et al., 1976), which interacts with cytoskeletal proteins and glycolytic enzymes (Low, 1986), and a 55 kDa carboxyl-terminal membrane domain (Steck et al., 1976). The membrane domain spans the lipid bilayer 12-14 times (Tanner, 1997) and is responsible for Cl^-/HCO_3^- exchange activity (Grinstein, Ship & Rothstein, 1978). The protein terminates with a cytoplasmic 33 amino acid carboxyl-terminal domain (Lieberman & Reithmeier, 1988; Wainwright et al., 1989). A truncated form of human AE1 beginning at methionine 66 is found in

1989). A truncated form of human AE1 beginning at methionine 66 is found in kidney (Kollert-Jons et al., 1993). Other plasma membrane anion exchange proteins include AE2 and AE3 and recently identified AE4, DRA and Pendrin (Alper et al., 1988; Kopito et al., 1989; Melvin et al., 1999; Rajendran et al., 2000; Soleimani et al., 2001; Tsuganezawa et al., 2001).

In mammals 14 CA isoforms have been identified (Kivela et al., 2000a; Mori et al., 1999; Tureci et al., 1998). Human erythrocytes express predominately CAI and a lesser amount of CAII (Maren, 1967). However, CAII accounts for the majority of carbonic anhydrase activity in human erythrocytes since it has a higher turnover rate (10^6 s^{-1}) (Maren, 1967) and CAI facilitated hydration of CO_2 is inhibited by 92% in physiological concentrations of Cl^- (80 mM) (Maren, Rayburn & Liddell, 1976). Interestingly, the erythrocyte contains approximately 10^6 copies of the CAII isoform (Tashian & Carter, 1976), which is stoichiometric with AE1 copies ($1.2x10^6$/ erythrocyte) (Ship et al., 1977; Steck et al., 1971). Effective Cl^-/HCO_3^- anion exchange in erythrocytes is dependent on the activity of cytosolic carbonic anhydrase (Cousin et al., 1975; Jacobs M.H. & D.R., 1942; Keilen D. & T., 1941; Maren & Wiley, 1970).

Several lines of evidence have shown an interaction between CAII and AE1. Binding of erythrocyte membranes to CAII has been show to increase its enzymatic activity (Parkes & Coleman, 1989). This interaction is weak, however, since the bulk of carbonic anhydrase can be readily removed from isolated erythrocyte membranes (Randall & Maren, 1972; Vince & Reithmeier, 1998). Reaction of an anion transport inhibitor (DIDS) with AE1 altered the binding of a fluorescent inhibitor to carbonic anhydrase suggesting a physical link between these two proteins (Kifor et al., 1993). Extracellular lectin caused agglutination of

AE1 and a similar redistribution of CAII on the cytosolic surface of the erythrocyte membrane (Vince & Reithmeier, 1998), suggesting a physical interaction of AE1 with CAII. CAII can be co-immunoprecipitated with solubilised AE1, and finally a sensitive microtiter assay showed that CAII but not CAI interacts with the C-terminus of AE1 (Vince & Reithmeier, 1998). Truncation and point mutation of the AE1 C-terminus, led to the identification of the binding site of CAII in human AE1 as LDADD (amino acids 886-890) (Vince & Reithmeier, 2000). Binding assays also showed that CAII interacts with the C-terminal region of AE2 (Vince & Reithmeier, 2000) but interaction between AE3 and CAII has not yet been examined. The interaction between AE1 and CAII is pH dependant (Vince & Reithmeier, 1998), which suggested binding of the acidic LDADD motif of AE1 with a basic region of CAII. Truncation and mutagenesis of the basic N-terminal region of CAII showed that it forms the AE1 binding site (Vince et al., 2000). Replacement of basic residues in the N-terminus of CAII with the equivalent residues in CAI resulted in a loss of AE1 binding (Vince et al., 2000). Truncation of the CAII N-terminal region also resulted in loss of binding ability but did not impair enzymatic activity, implying that the function of the basic N-terminal domain is to bind CAII to AE1, or other proteins with similar acidic binding motifs.

In this study we tested the functional consequences of the AE1/CAII interaction. Our hypothesis is that this interaction facilitates the coupling of CAII enzymatic activity and anion exchange activity, resulting in more efficient bicarbonate transport. Using HEK 293 cells transiently transfected with AE1 cDNA, we determined that inhibition of endogenous CAII activity with acetazolamide resulted in a decrease of AE1 transport activity. Mutation of the

AE1 LDADD acidic binding motif to LAAAA or LNANN caused a loss of CAII binding and also a decrease of AE1 transport activity. Binding of functionally inactive V143Y CAII mutant (Fierke et al., 1991) had a dominant negative effect on anion transport. Over-expression of V143Y CAII also caused a reduction of AE2 and AE3c transport activity. This first demonstration of a functional interaction between CAII and AE3c leads us to conclude that binding of CAII to the C-terminus of AE proteins is required for maximal transport activity. The requirement of a physical interaction between CAII and AE1 for maximal bicarbonate transport activity suggests that the AE1/CAII complex forms a functional transport metabolon: a physically associated complex of proteins in a sequential metabolic pathway (Srere, 1985; Srere, 1987).

4.2 Results

4.2.1 Expression of CAII and AE1 in HEK 293 cells

Functional studies were performed in transiently transfected HEK 293 cells. These cells express practically undetectable levels of chloride/bicarbonate exchange activity (Kopito et al., 1989) but do contain endogenous carbonic anhydrase. All of the cDNAs were inserted into the pcDNA3.1 vector or pRBG4 (Lee et al., 1991), which placed them under the control of the human cytomegalovirus early gene promoter. Immunoblots of lysates from transfected HEK 293 cells were probed with either a polyclonal antibody directed against the carboxyl-terminal region of AE1 or an anti-CAII antibody. Figure 4.1 indicates that wild-type AE1 and the LAAAA and LNANN mutants were expressed at similar levels in transfected HEK 293 cells. Cells transfected with vector alone showed no immunoreactivity for AE1. Immunoblots using an anti-CAII

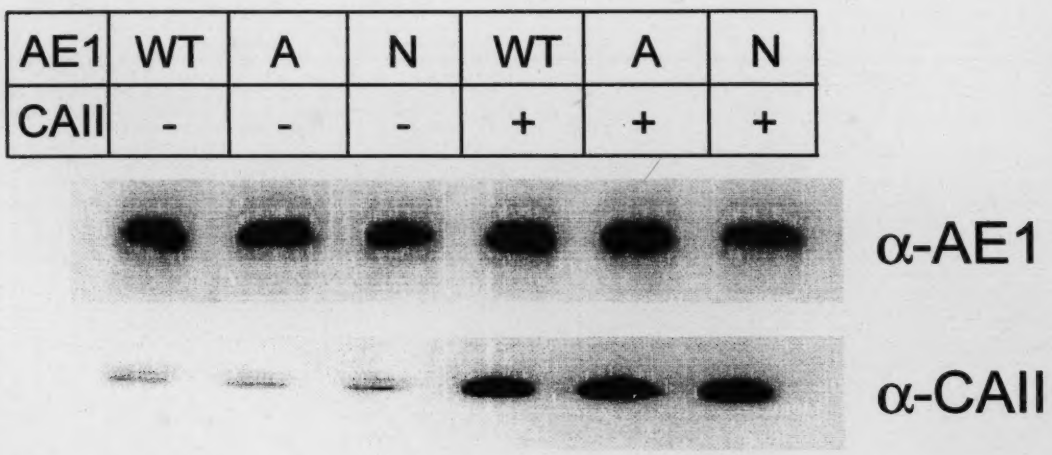

Figure 4.1 Expression of AE1 and CAII in transfected cells.

HEK 293 cells were transiently transfected with cDNA coding for wild-type AE1 (WT), LAAAA mutant of AE1 (A), LNANN mutant of AE1 (N), with or without (+ or -) co-transfection with cDNA for wild-type CAII. Two days post transfection, cells were solubilised. Samples (5 µg protein) were resolved by SDS-PAGE on 8% (samples probed for AE1) or 12.5 % (samples probed for CAII) acrylamide gels and transferred to PVDF membrane. Immunoblots were probed with either a rabbit polyclonal antibody directed against the C-terminus of human AE1 (α-AE1) or sheep-anti human CAII antibody (α-CAII).

antibody showed that HEK 293 cells express CAII and could also be transfected with cDNA for human CAII to increase the level of this enzyme. Densitometry of immunoblots indicated that CAII was over-expressed approximately 20-fold compared to endogenous CAII found in HEK 293 cells. Absolute amounts of AE1 and CAII expression were determined by immunoblot comparison of expression in transfected HEK 293 cells to known amounts of human erythrocyte AE1 protein and purified CAII.The stoichiometry of AE1:CAII was 1.7:1 in cells transfected with AE1-alone or 1:12 in CAII and AE1 co-transfected cells. The same amount of endogenous CAII was expressed in sham-transfected cells and cells transfected with AE1-alone.

4.2.2 Effect of CAII inhibition on Cl^-/HCO_3^- exchange activity

To determine the role of CAII in facilitating AE1 transport activity, we compared the Cl^-/HCO_3^- exchange activity of cells transiently transfected with AE1 cDNA before, and after incubation with acetazolamide, a sulfonamide that inhibits CA enzymatic activity without direct effect on anion exchange (Cousin & Motais, 1976; Cousin et al., 1975). To measure AE1 transport activity, HEK 293 cells, grown on coverslips were transiently transfected with AE1 cDNA, loaded with BCECF-AM, a pH sensitive fluorescent dye, and mounted in a fluorescence cuvette. Cells were perfused alternately with chloride-containing (140 mM), and chloride-free Ringer's buffer. Solutions at pH 7.4 were continually bubbled with 5% CO_2 in balanced air (P_{CO2} = 40 mm Hg) resulting in a constant extracellular bicarbonate concentration of 25 mM (Zeidel, Silva & Seifter, 1986). In chloride-free Ringer's buffer, Cl^- will leave the cell in exchange for extracellular HCO_3^- causing cell alkalinisation. The reverse happens when cells are perfused with

chloride-containing buffer. BCECF fluorescence changes with pH and with appropriate calibration will give a measurement of changes in intracellular pH (pH_i). Previous determination of the intrinsic buffering capacity of HEK 293 cells (Lee et al., 1991; Sterling & Casey, 1999) enables the rate of change in pH_i to be converted to H^+ flux. The intrinsic buffering capacity was negligible above pH 7.1, thus H^+ flux was determined as the product of the change in pH/time and bicarbonate buffering (2.3 X $[HCO_3^-]$) (Roos & Boron, 1981). The typical rise of intracellular pH from pH 7.2 to 7.7 in these assays corresponds to a rise of intracellular bicarbonate of 35 mM. This rise occurs readily in exchange for efflux of intracellular Cl^-, which is found in mammalian cells at 25-60 mM (Pilas & Durack, 1997). In these assays, transport rates were determined by linear regression of the initial slopes of curves produced as pH_i changes. AE1 transport activity was measured and then cells were incubated for 10 minutes with acetazolamide. As acetazolamide will not covalently react with the CA, all buffers used subsequent to the incubation also contained the appropriate concentration of acetazolamide. This allowed for comparison of anion transport activity of the same population of cells in the absence and presence of acetazolamide.

The effect of acetazolamide on anion exchange activity was monitored during both bicarbonate influx (Cl^--free Ringer's buffer) and during bicarbonate efflux (Cl^--containing Ringer's buffer). In Figure 4.2A acetazolamide (100 µM) was initially added to transfected HEK 293 cells in Cl^--free Ringer's buffer, which caused inhibition of both AE1-mediated bicarbonate influx and efflux. However the two transport rates were not equally inhibited; bicarbonate influx was inhibited by 49 ± 5%, while bicarbonate efflux was inhibited by 62 ± 3%. The

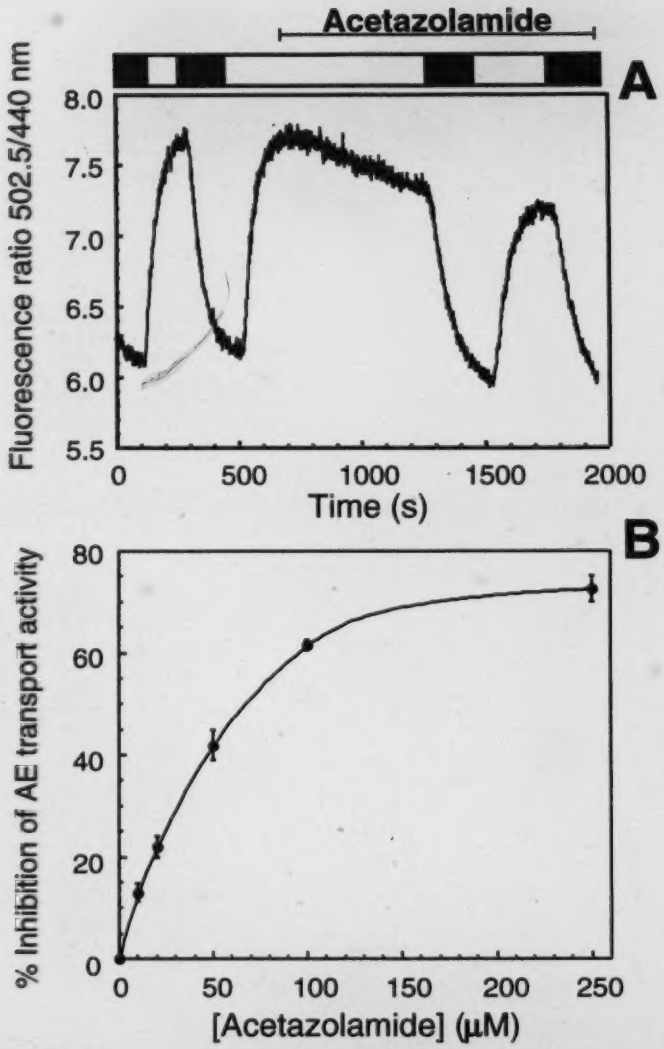

Figure 4.2 Full AE1 transport activity requires active carbonic anhydrase.
HEK 293 cells transfected with AE1 cDNA were loaded with BCECF-AM and placed in a fluorescence cuvette in a fluorimeter. Cells were perfused alternately with Cl^--containing (solid bar) and Cl^--free (open bar) Ringer's buffer and fluorescence was monitored using excitation wavelengths 440 and 502.5 nm and emission wavelength 528.7 nm. Cells were then incubated with acetazolamide for 10 minutes followed by a repeat of the Ringer's buffer perfusion. Transport activity following acetazolamide incubation was compared to that before the incubation, and expressed as a percentage of this transport activity. A, effect of 100 μM acetazolamide on AE1 mediated HCO_3^- transport. B, Relative degree of inhibition of AE1-mediated bicarbonate efflux following acetazolamide incubation. Standard error bars are indicated (n=4).

larger inhibition of bicarbonate efflux caused by acetazolamide was statistically significant ($p<0.05$; $n=4$).

Figure 4.2B is a dose-response curve for the effect of acetazolamide (added initially in Cl^--free Ringer's buffer) on bicarbonate efflux. AE1 bicarbonate efflux activity was maximally inhibited by 70 ± 2% at an acetazolamide concentration of 250 μM. The apparent Ki was 54 μM, which is higher than the 10^{-8} M value measured using purified CAII (Maren, 1967). The concentration of carbonic anhydrase inhibitors required to have an effect on anion exchange in erythrocytes were also found to be several orders of magnitude higher than required to inhibit CAII (Cousin et al., 1975; Maren & Wiley, 1970). In these studies, CAII did not become rate limiting for anion exchange until the enzyme was inhibited to greater than 99%. This is because of the high activity of CAII relative to the transport activity of AE1 in red cells, requiring doses of acetazolamide in the mM range. Acetazolamide does not directly inhibit AE transport activity, as it cannot inhibit chloride/bicarbonate (Cousin et al., 1975) or chloride/chloride or oxalate self exchange (Cousin & Motais, 1976). Thus, the observed decrease in AE transport activity in the presence of acetazolamide was due to the inhibition of CA activity.

4.2.3 Cl^-/HCO_3^- exchange activity of AE1 mutants

To determine whether the interaction of CAII with AE1 was necessary for optimal transport, the transport mediated by two different mutants of the AE1 CAII binding site (LNANN, LAAAA) was determined. Neither of these mutants is able to bind CAII (Vince & Reithmeier, 2000). HEK 293 cells, transiently transfected with cDNA encoding wild-type, LAAAA or LNANN AE1, were

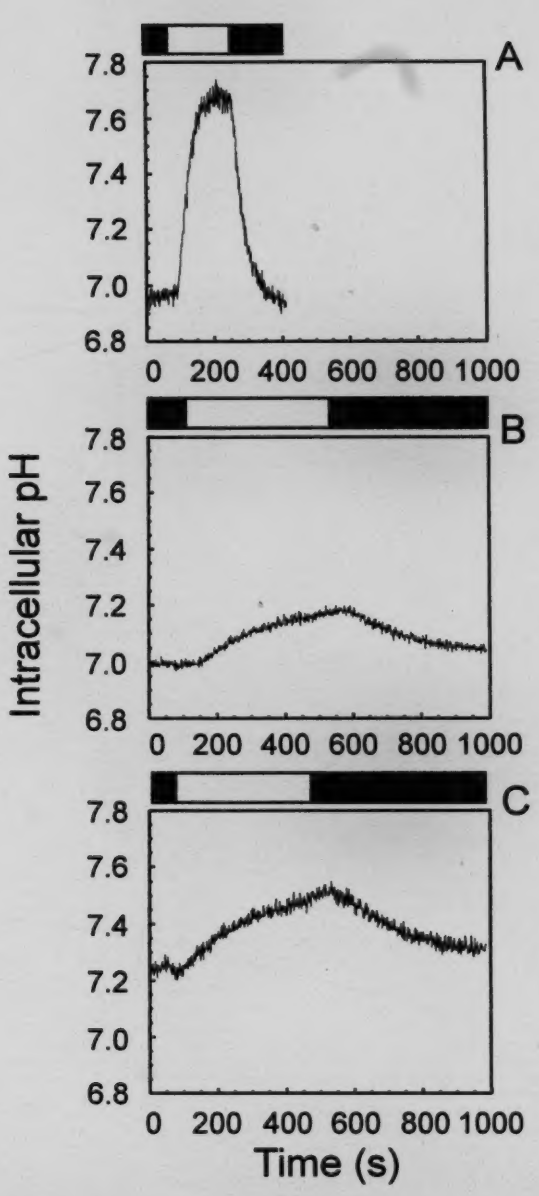

Figure 4.3 Transport activity of AE1 mutant proteins unable to bind CAII.
HEK 293 cells transiently transfected with AE1 cDNA only were loaded with BCECF-AM. Cells were perfused alternately with Cl^--containing (solid bar) and Cl^--free (open bar) Ringer's buffer and fluorescence monitored using excitation wavelengths 440 and 502.5 nm and emission wavelength 528.7 nm. Transport activity of HEK 293 cells transfected with A, wild-type AE1, B, LAAAA mutant AE1 and C, LNANN mutant AE1.

subjected to the transport assay described above. Figure 4.3 shows typical anion exchange assays. Wild-type AE1 had a transport rate of about 10 times that of the mutant LAAAA and LNANN AE1 proteins (40 ± 0.6 versus 4 ± 0.6 and 3 ± 0.6 mM H^+ equivalent/min, respectively). The two mutants of the AE1 C-terminal tail thus clearly had reduced transport rates relative to wild-type.

The large difference in transport activity between the wild-type and mutant AE proteins may be due to differences in protein expression at the plasma membrane. The total amount of expression of wild-type AE1 and the two mutants was similar (Figure 4.1). The activity assay used to measure transport rate, only measures the activity of protein expressed at the plasma membrane, thus any protein retained intracellularly is observed as non-functional. To address the possibility that introducing mutations intothe protein interferes with the ability of the protein to be properly processed to the plasma membrane, we investigated the amount of expressed protein present in the plasma membrane for each of the AE transport proteins investigated by cell surface biotinylation. The fraction of protein expressed at the plasma membrane was similar for wild-type and LNANN mutants (32 ± 2 and 37 ± 3%, respectively; n=5) and statistically higher for the LAAAA mutant (61 ± 3%) than for wild-type AE1 ($p<0.0002$, n=5). Thus, the lower activity of the two C-terminal mutants of AE1 is not explained by reduced expression or processing to the cell surface.

4.2.4 Over-expression of CAII in HEK 293 cells

Since the stoichiometry of AE1/CAII in AE1 transfected HEK 293 cells was 1.7:1, we determined whether CAII activity was rate limiting to measured AE transport activity. CAII was co-expressed with the AE proteins by transient

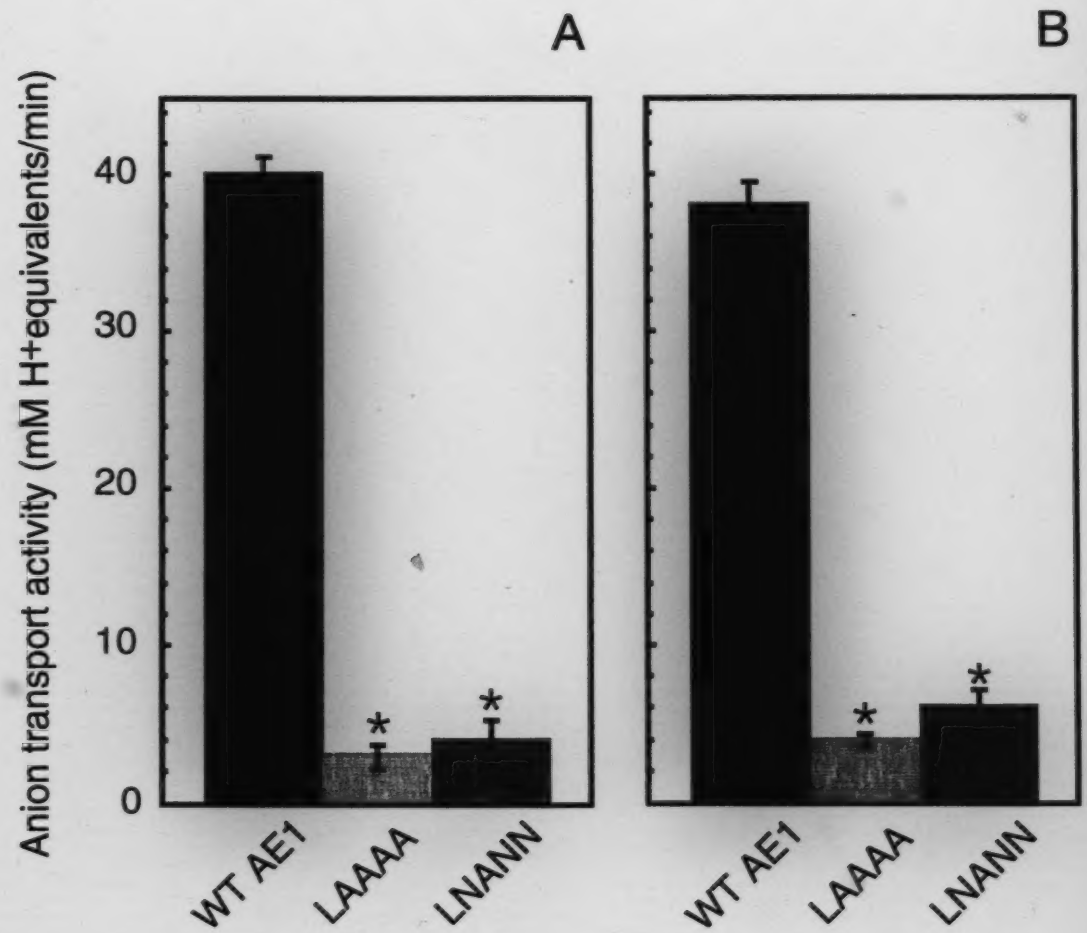

Figure 4.4 Summary of effect of AE1 C-terminal mutations.

A, transport activity of cells transiently transfected with AE1 cDNA. B, transport data collected for cells co-transfected with AE1 and CAII cDNAs. Standard error bars are also indicated (n=6) and asterisks denote a significant difference in transport rates ($p<00001$) relative to wild-type AE1.

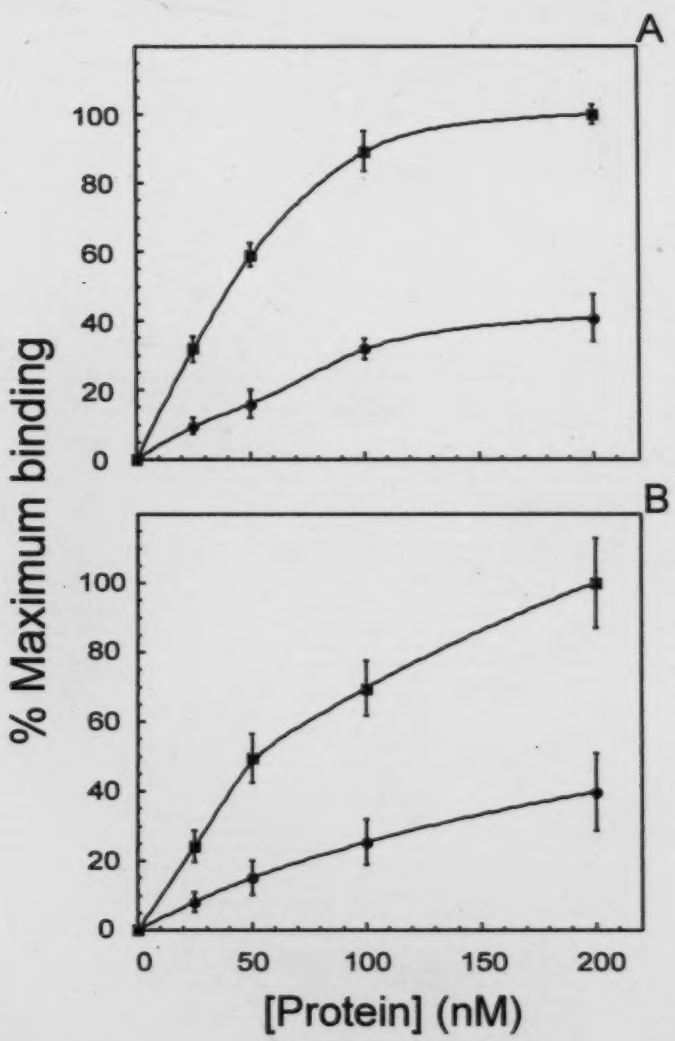

Figure 4.5 CAII and mutant V143Y bind to the carboxyl-terminal tail of AE1.
The binding of GST-AE1ct and GST to immobilised CAII and an inactive CAII (V143Y) was measured using a microtiter plate assay. Wild-type CAII (Panel A) and CAII V143Y (Panel B) were immobilised on 96 well microtiter plates. The immobilised CAII was incubated with different concentrations of GST-AE1ct (squares) and GST alone (circles). Bound proteins were detected by incubation of plates sequentially with goat anti-GST antibody, biotinylated rabbit anti-goat IgG and then peroxidase-labeled biotin/avidin. This was followed by incubation with substrate o-phenyldiamine and detection of enzymatic activity at 450 nm in a microplate reader. Quadruplicate measurements for each concentration of protein were made on the same plate. Error bars represent standard error of the mean (n =4).

transfection, and transport activity was also determined. Figure 4.4 summarises the transport activity of the AE proteins when expressed alone or along with excess CAII. Co-transfection with wild-type CAII cDNA had no significant effect on transport activity of any of the AE1 constructs, confirming that the endogenous amount of CAII present in HEK 293 is not rate limiting to AE transport activity. In addition, over-expression of CAII was not able to rescue the binding defect of the LAAAA or LNANN mutants.

4.2.5 Effect of CAII V143Y on AE1 Cl^-/HCO_3^- exchange activity

The above experiments suggest that binding of CAII to the C-terminus of AE1 is required for full AE1 activity. Another experiment to test this hypothesis was to co-express a functionally inactive CAII mutant (Fierke et al., 1991), with AE proteins. Over-expression of the CAII V143Y mutant should compete with the endogenous wild-type CAII for binding to the AE1 C-terminus. To assess the relative binding affinities of CAII and V143Y CAII for the AE1 C-terminus, CAII proteins were immobilised on microtiter dishes and incubated with varied concentrations of either GST or GST fused to the C-terminal 33 amino acids of AE1 (GST-AE1ct). Figure 4.5 shows that wild-type CAII and the V143Y mutant bind GST-AE1ct with similar affinities. Binding of GST-AE1ct to both CAII proteins is much higher than observed for GST alone.

To assess the effect of V143Y CAII on AE1 transport activity, HEK 293 cells were transfected with a range of amounts of CAII cDNA and a fixed amount of AE1 cDNA. Figure 4.6 shows that the level of AE1 expression does not change with varied CAII expression. The level of expression of CAII and V143Y CAII increased with increasing amounts of their respective cDNAs (Figs 1 and 6).

Expression of increasing amounts of wild-type CAII protein had no effect on AE1 transport activity, confirming that the endogenous level of CAII was sufficient for effective anion exchange (Figure 4.6). However, increasing the amount of expression of V143Y CAII, while keeping the amount of AE1 protein constant, decreased AE1 transport rates by up to 60%. These results suggest that the inactive CAII mutant displaces active endogenous CAII from its AE1 binding site. As increasing amounts of inactive CAII are expressed, the corresponding decrease in transport rate implies that binding of functional CAII to AE1 is required for maximal transport activity.

4.2.6 CAII interaction is required by AE2 and AE3

The data presented suggest that binding of CAII at the AE1 C-terminus is essential for maximal bicarbonate transport activity. Does this requirement extend to other chloride/bicarbonate exchangers? Figure 4.7 is an alignment of the C-terminal sequences of identified bicarbonate transporters. Some form of the hydrophobic residue/acidic sequence CAII binding motif is observed for AE1, AE2, AE3, a novel anion exchanger termed AE4 and Pendrin but not DRA. The sodium-dependent bicarbonate transporters NBC1b, NBC3 and NDCBE1 all also contain a consensus binding site, suggesting that CAII binding might also be required for some other bicarbonate transporters. To test this possibility, AE2 and AE3c were co-expressed along with wild-type and mutant CAII. AE3c is a variant of AE3, which differs from full-length AE3 at its extreme cytoplasmic N-terminus (Yannoukakos et al., 1994). Figure 4.8 indicates that, although over-expression of wild-type CAII had no significant effect on the transport rates of these two proteins, expression of the V143Y CAII impaired their transport

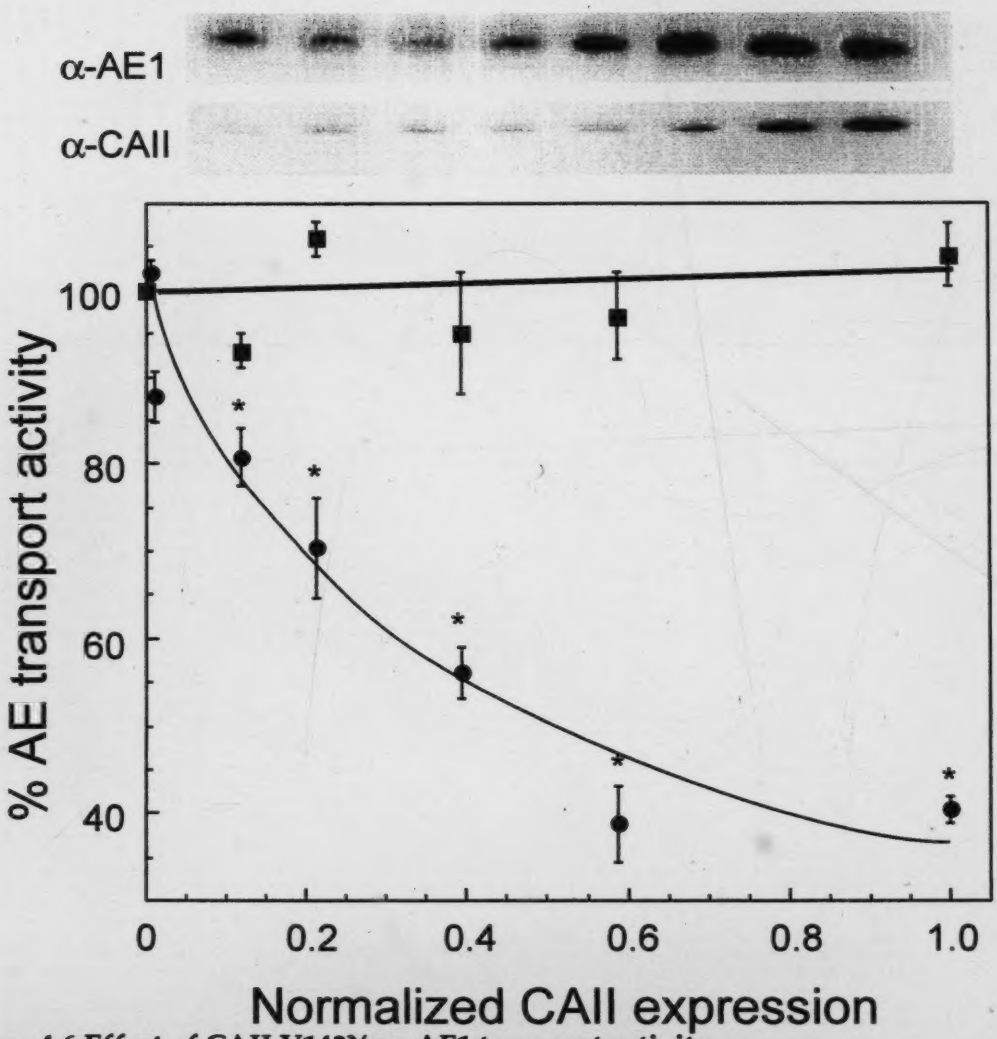

Figure 4.6 Effect of CAII V143Y on AE1 transport activity.

HEK 293 cells grown on coverslips were transiently co-transfected with cDNA encoding for AE1 (3.8 μg) and increasing amounts of either wild-type CAII, or V143Y CAII. Immunoblots indicate the expression of AE1 (top panel) with increasing amounts of V143Y CAII (0, 0.1, 0.2, 0.5, 1.0, 2.0, 3.8 μg cDNA) (middle panel). Two days post transfection cells were subjected to anion exchange assays. Transport rates are expressed relative to rate for AE1 expressed alone. Transport activity of cells co-transfected with AE1 and wild-type CAII (squares) or V143Y CAII (circles) are shown (bottom panel). Asterisks indicate a significant difference ($p<0.05$) between activity in the presence of wild-type CAII and V143Y CAII. Standard error bars are also indicated (n=6). Curve for wild-type CAII was fitted by linear regression and V143Y CAII data was fitted manually.

hAE1	RNVELQCLDADDAKATFDEEEGRDEYDEVAMPV
hAE2	TDREMKCLDANEAEPVFDEREGVDEYNEMPMPV
hAE3	QDRELQALDSEDAEPNFDEDGQDEYNELHMPV
rAE4	SPQELLWLDELMPEEERNVPEKGLEPGHSFSGSDSEDSELMYQPKAPEINISVN
hNBC1b	SQHDLSFLDDVIPEKDKKKKEDEKKKKKKKGSLDSDNDDSDCPYSEKVPSIKIP
	MDIMEQQPFLSDSKPSDRERSPTFLERHTSC
hNBC3	TKRELSWLDDLMPESKKKKEDDKKKKEKEEAERMLQDDDDTVHLPFEGGSLLQ
	IPVKALKYSPDKPVSVKISFEDEPRKKYVDAETSL
hNDCBE1	SKRELSWLDDLTPESKKKKLDDAKKKAKEEEVIV
hPendrin	LEELDIPTKEIEIQVDWNSELPVKVNVPKVPIHSLVLDCGAISFLDVVGVRSLRVIV
	KEFQRIDVNVYFASLQDYVIEKLEQCGFFDDNIRKDTFFLTVHDAILYLQNQVKSQ
	EGQGSILETITLIQDCKDTLELIETELTEEELDVQDEAMRTLAS
hDRA	KKDYSTSKFNPSQEKDGKIDFTINTNGGLRNRVYEVPVETKF

Figure 4.7 Alignment of amino acid sequences of putative cytoplasmic, C-termini of bicarbonate transport proteins.

Potential CAII binding sites (grey box), consisting of a hydrophobic residue followed by a short cluster containing 2-3 acidic residues, are indicated. The first letter of each sequence name refers to the species: h (human) and r (rabbit). AE1, (Tanner et al., 1988); AE2, (Gehrig et al., 1992); AE3, (Yannoukakos et al., 1994); AE4a (Tsuganezawa et al., 2001); NBC1b (Choi et al., 1999); NBC3 (Pushkin et al., 1999); NDCBE1 (Grichtchenko et al., 2001); Pendrin, (Baldwin et al., 1995); DRA, (Melvin et al., 1999). Cytoplasmic tail sequences were identified by homology with AE1. However, the sequences of DRA and Pendrin were sufficiently different from the other proteins that the cytoplasmic tail sequences could not be confidently identified. Sequences shown represent C-terminal hydrophilic regions.

activity by maximally 40%. This suggests that interaction with CAII may be required for full bicarbonate transport activity of many or all anion exchange proteins.

4.3 Discussion

Anion exchange proteins and CAII are together responsible for bicarbonate transport and metabolism. Chloride/bicarbonate anion exchange in erythrocytes is dependent for its full effectiveness on CAII activity (Cousin et al., 1975; Jacobs M.H. & D.R., 1942; Keilen D. & T., 1941; Maren & Wiley, 1970). CAII provides the substrate for bicarbonate efflux by AE1 and converts the bicarbonate that enters via AE1 to CO_2 and H_2O. Early studies suggested that AE1 and CAII form a complex (Kifor et al., 1993). The CAII binding site on AE1 was recently identified as the acidic motif LDADD in the C-terminal tail of AE1(Vince & Reithmeier, 1998; Vince & Reithmeier, 2000). However, the effect of binding of CAII to the C-terminus of AE1 upon transport activity was unknown. In the present report, we have examined the relationship between CAII functional activity and plasma membrane chloride/bicarbonate exchange activity. Our data showed that inhibition of CAII by acetazolamide maximally impaired AE1 transport activity by 70 ± 2%, indicating that CAII activity was required for optimal chloride/bicarbonate exchange activity. Two mutants of AE1, shown previously to be unable to bind CAII (Vince & Reithmeier, 2000), had anion exchange activity barely above background (10% of wild-type AE1 activity). The defect in transport activity of these mutants was shown not to be due to reduced expression or processing to the cell surface. Taken together these data suggest

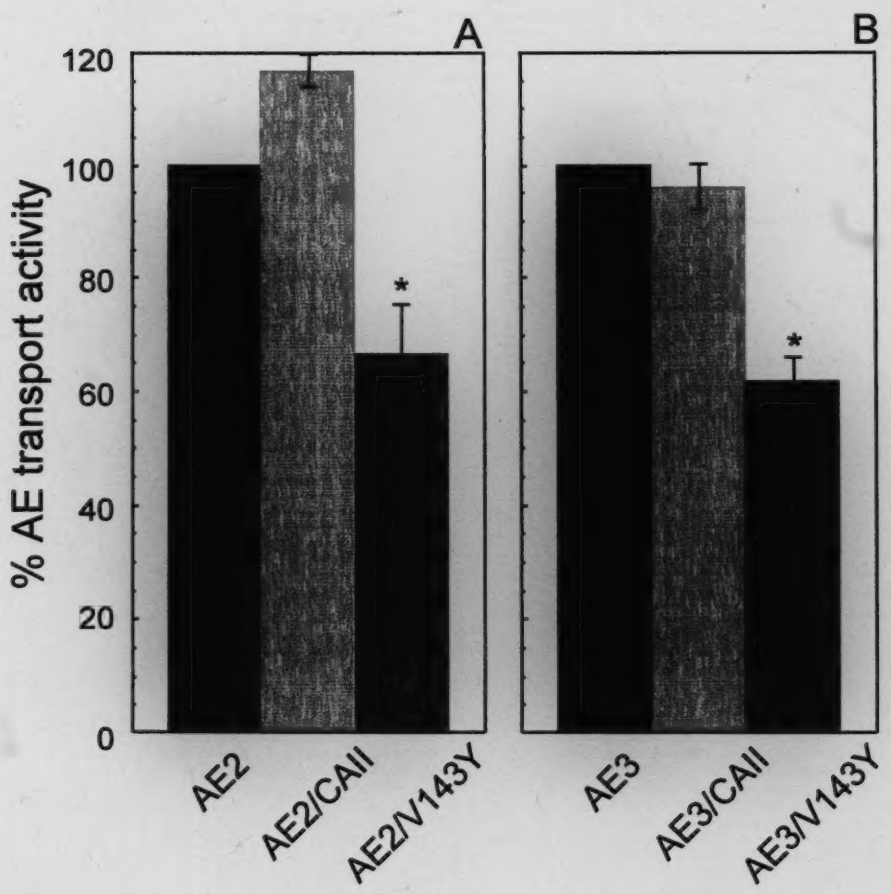

Figure 4.8 Effect of CAII V143Y on AE2 and AE3c transport activity.

HEK 293 cells were transiently co-transfected with AE2 or AE3c cDNA (3.8 μg) and 3.8 μg of either wild-type or V143Y CAII cDNA and anion exchange activity was measured. A, relative transport activity of cells transiently transfected with AE2 cDNA alone, and with CAII isoforms as indicated. B, relative transport activity of cells transiently transfected with AE3c cDNA alone, and with CAII isoforms as indicated. Standard error bars are also indicated (n=4) and asterisks denote a significant difference in transport rates ($p<0.05$) relative to transport rate of AE protein expressed alone.

that direct interaction with CAII is required for full anion exchange activity by AE1.

The most definitive experiment leading to the above conclusion was the co-expression in HEK 293 cells of the functionally inactive V143Y CAII mutant along with AE1, in the presence of endogenous CAII. Data presented here showed that wild-type CAII and the V143Y mutant bind the AE1 C-terminus with equal affinity (Figure 4.5). We found that increasing levels of V143Y CAII expression proportionately inhibited AE1 transport activity, to a maximum level of 60% inhibition. The level of inhibition is similar to the effect of inhibition of CAII by acetazolamide on anion transport activity. This result would be observed if CAII expression reduced the level of AE1 expression. However, over-expression of wild-type CAII did not affect AE1 activity. In addition, when the level of AE1 expression was measured on immunoblots, the level of AE1 expression was constant at all levels of V143Y CAII expression. We therefore interpret the data from the V143Y experiment as follows. HEK 293 cells transfected with CAII cDNA over-express the protein about 20 times the level of the endogenous protein. Thus, the introduced CAII effectively will displace endogenous CAII from its binding site on AE1. In the case of cells transfected with wild-type CAII this has no effect on AE1 activity since the level of CAII is not rate-limiting to transport and introduced CAII is as catalytically active as the endogenous CAII. However, when V143Y CAII is over-expressed, it will displace the endogenous wild-type CAII from its binding site on AE1. Under these conditions, we observed a substantial decrease in AE1 transport activity. Since the total amount of functional CAII activity is constant between cells transfected with V143Y CAII and cells transfected with AE1 alone, we conclude

that the decrease of AE1 transport activity is due to displacement of CAII from the C-terminal tail of AE1. A similar dominant negative effect of V143Y CAII was found for transport activity by the AE2 and AE3c anion exchange proteins. We conclude that binding of CAII to the C-terminal tail of AE1 is essential for maximal AE transport activity. This finding may generalise to all bicarbonate transporters since CAII binding to both AE2 and AE3c is required for full function of these proteins as well. Modulation of the interaction of CAII with anion exchangers would provide a powerful mechanism to regulate bicarbonate transport.

The stoichiometry of AE1 and CAII expression in transfected cells is informative. We found that, in AE1-transfected cells the total AE1: endogenous CAII stoichiometry was 1.7:1, while co-transfection of HEK 293 cells with AE1 and CAII caused the stoichiometry to change to 1:12. Since our data shows that only 32% of AE1 is processed to the cell surface, if we assume that all CAII is localised to the plasma membrane, this implies a plasma membrane associated AE1/CAII stoichiometry of 1:1.8, in cells not transfected with CAII. In erythrocytes, the stoichiometry is very close to 1:1 as discussed above. At 37 °C the turnover rates for AE1 (with Cl^- as substrate) and for CAII are respectively 5 x 10^4 s^{-1} (Jennings, 1989) and 10^6 s^{-1} (Maren, 1967), respectively. Taken together, in AE1-alone transfected HEK 293 cells there is approximately 36 fold more carbonic anhydrase activity than anion exchange activity and in erythrocytes there is approximately 20 fold more CAII activity than anion transport activity. Therefore, both in AE1-transfected HEK 293 cells and erythrocytes, anion transport activity is rate limiting to the transmembrane bicarbonate flux. The

assay used to assess bicarbonate transport rate, in transfected HEK 293 cells, uses the rate of change of intracellular pH induced by transmembrane bicarbonate transport as a measure of bicarbonate flux. This is valid since the rate of CO_2/bicarbonate conversion is much faster than the rate of bicarbonate transport.

Three different methods were used to alter carbonic anhydrase activity, or localisation in HEK 293 cells: inhibition with acetazolamide, mutation of the AE1 C-terminal region and over-expression of V143Y CAII. Each of these methods inhibited AE1 transport activity to differing extents: 90% inhibition was seen in the AE1 mutants, while 60-70% inhibition was induced by acetazolamide or the V143Y CAII dominant-negative mutant. The larger effect seen by mutation of the AE1 C-terminus may reflect the complete abolition of CAII binding caused by the mutation. However, we cannot rule out the possibility that these mutants may be functionally compromised in some way unrelated to CAII binding.

At 250 µM acetazolamide, maximal reduction of observed transport activity was 70% and the Ki for the effect of acetazolamide on observed AE1 activity was 54 µM, much higher than the Ki (10 nM) for the effect of acetazolamide on CAII activity (Maren, 1967). This observation may be due to the fact that endogenous carbonic anhydrase activity of AE1-transfected HEK 293 cells is much higher than the total anion exchange activity. Thus no reduction of anion exchange activity measured in our assay would be expected until CAII was nearly completely inhibited, as found in the red cell system (Cousin & Motais, 1976; Maren & Wiley, 1970).

Over expression of V143Y CAII may not inhibit anion exchange as potently as mutation of the AE1 C-terminus for two reasons. V143Y CAII has

3000 fold lower catalytic activity than wild-type CAII (Fierke et al., 1991). Although this is a great reduction, the catalytic rate of approximately 300 s^{-1} remains. Second, CAII V143Y is over-expressed 20 fold over the level of endogenous wild-type CAII. Thus, approximately 5% of CAII molecules bound to AE1 would be expected to be wild-type. Together with the low activity of the V143Y mutant, this likely accounts for the residual AE1 activity observed.

Data presented here supports the idea that the complex of AE1 and CAII forms a bicarbonate transport metabolon (Reithmeier, 2001; Vince & Reithmeier, 1998; Vince & Reithmeier, 2000). A metabolon is a complex of enzymes involved in a linked metabolic pathway that allows metabolites to move easily from one active site to the next (Srere, 1985; Srere, 1987), a process also called channeling. Known enzymatic metabolons include the enzymes of the glycolysis, citric acid and urea cycles (Reithmeier, 2001). Channeling allows for increased flux through the pathway as it limits the loss of intermediates by diffusion (Miles, Rhee & Davies, 1999). In addition, a metabolon would allow the creation of specific pools of substrates. Since AE1 can transport a range of small anions, localisation of CAII effectively concentrates bicarbonate at the transport site, favoring its transport over other substrates in the cell. The presence of similar numbers of AE1 and CAII copies in the erythrocyte (Ship et al., 1977; Steck et al., 1971; Tashian & Carter, 1976) provided the initial support for the theory of a metabolon complex between AE1 and CAII. Our observation of decreased transport activity upon disruption of the CAII/AE1 interaction by mutagenesis, or over-expression of V143Y CAII, provides functional evidence for a bicarbonate transport metabolon. Figure 4.9 is a model that illustrates acceleration of bicarbonate transport by a bicarbonate transport metabolon. The model

illustrates that localisation of CAII to the AE1 C-terminus maximises the local concentration of bicarbonate at the transport site of AE1 during bicarbonate efflux. Conversely, during bicarbonate influx CAII minimises the local concentration of bicarbonate, by conversion to carbon dioxide. Thus, the binding of CAII to AE1 localises the enzyme to the cytosolic surface of the membrane where it can facilitate CO_2 movement across the lipid bilayer.

We compared the Cl^-/HCO_3^- exchange activity of AE1-transfected cells before, and after incubation with acetazolamide. In anion exchange assays cells were alternately perfused with Cl^--free and Cl^--containing Ringer's buffer. Thus, AE1 transported Cl^- out of or into the cell, down a concentration gradient. In exchange, bicarbonate was transported into or out of the cell, in a coupled one for one exchange process. Acetazolamide (100 μM) inhibited AE1-mediated bicarbonate influx by 49 ± 5% and efflux by 62 ± 3%, which was a statistically significant difference. We have shown that maximal AE activity is observed when CAII directly associates with the AE1 C-terminus. As illustrated by Figure 4.9, the difference in acetazolamide effect on bicarbonate efflux rate versus influx rate reflects the fact that during bicarbonate efflux, diminished transport will occur in the absence of CAII, because CAII produces the bicarbonate substrate for efflux. The transport rate directly depends on the local bicarbonate concentration, produced by CAII. During bicarbonate influx, bicarbonate is present at 25 mM on the extracellular face, irrespective of CAII. The acceleration of transport observed in the presence of CAII is due to the consumption of influxed bicarbonate. While, this accelerates bicarbonate transport, the effect is not quite as large as CAII action is during efflux.

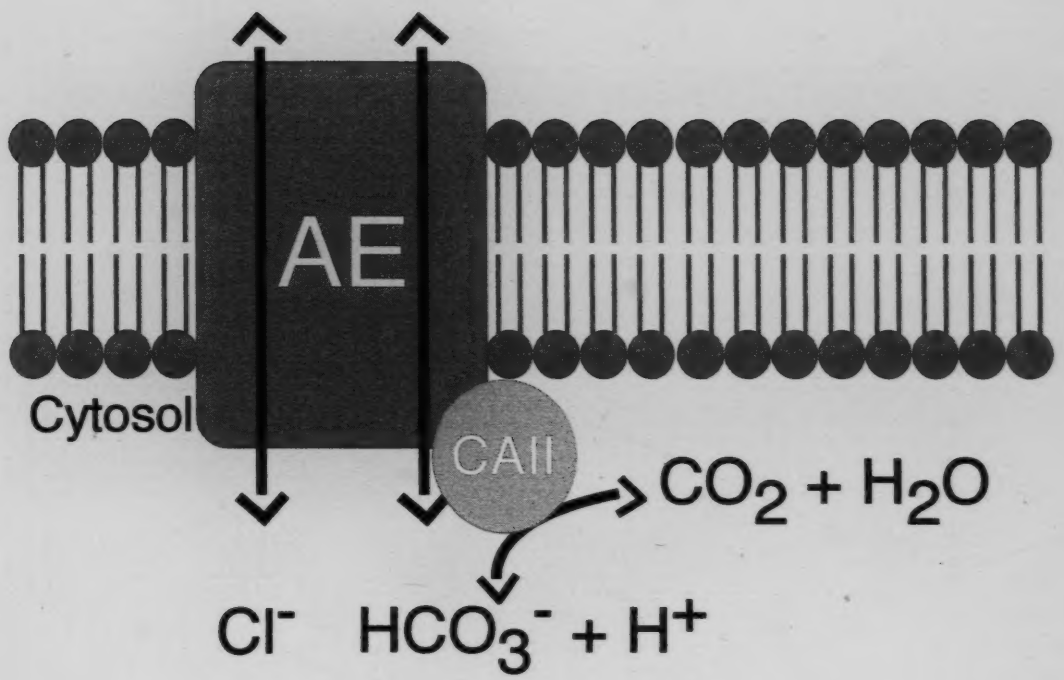

Figure 4.9 A bicarbonate transport metabolon.

Anion exchangers (AE) facilitate the reversible exchange of Cl^- for HCO_3^- across the plasma membrane. AE anion exchange proteins (AE1, AE2, and AE3) require binding of CAII to their carboxyl-termini to ensure maximal anion transport activity. Free CAII in the cytosol is not sufficient to support full AE transport function. The complex of CAII and AE maximises the bicarbonate transport rate by minimising the distances of substrate diffusion between CAII and AE. Regulation of the interaction could therefore modulate the transport rate.

Several other bicarbonate transport proteins have recently been identified, including AE4, DRA, Pendrin and sodium-bicarbonate co-transporters (NBC). AE4 was recently identified in apical membranes of β-intercalated cells in the kidney and functionally characterised (Tsuganezawa et al., 2001). Cl^-/HCO_3^- exchange function in the human colon and ileum has been attributed to the protein downregulated in adenoma (DRA) (Melvin et al., 1999; Rajendran et al., 2000). Pendrin is expressed in the thyroid gland (Scott et al., 1999), ear (Everett et al., 1999) and kidney (Soleimani et al., 2001). Expression in HEK 293 cells demonstrated that Pendrin can function as a Cl^-/OH^-, Cl^-/HCO_3^-, and Cl^- /formate exchanger (Soleimani et al., 2001). Sodium/bicarbonate co-transporters have been characterised in the kidney (Boron & Boulpaep, 1989; Burnham et al., 1997). Sequence alignment of the extreme C-terminal tail of bicarbonate transport proteins (Figure 4.7) indicates that some of these proteins also have potential CAII binding sites. It is therefore possible that interaction with CAII maximises transport activity of many or all bicarbonate transporters.

We have presented evidence for a transport metabolon linking a cytosolic enzyme to a transporter. This physical interaction facilitates the movement of substrate from one through the other. Our study clearly demonstrates that AE1, AE2 and AE3 all require CAII binding at their C-terminus for maximal bicarbonate transport activity; sequence alignments suggest interaction with CAII could be important for a host of bicarbonate transporters. The large effect of CAII binding on transport activity also suggests an effective mechanism for regulation of bicarbonate transport. Modulation of the CAII/ transport protein interaction, for example by phosphorylation, would profoundly influence the rate of bicarbonate transport. Interestingly, hypertonic treatment of human

erythrocytes causes phosphorylation of Y904, adjacent to the CAII binding site (Minetti et al., 1998; Yannoukakos et al., 1991). Recruitment of CAII to the C-terminal tail of anion exchangers could provide a mechanism to increase chloride/bicarbonate exchange activity.

Chapter 5

The Functional and Physical Relationship Between the Downregulated in Adenoma Bicarbonate Transporter and Carbonic Anhydrase II[4]

[4]**Portions of this chapter have been accepted for publication:**
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The Functional and Physical Relationship Between the Downregulated in Adenoma Bicarbonate Transporter and Carbonic Anhydrase II

5.1 Introduction

Bicarbonate metabolism is essential in humans, since carbon dioxide is the metabolic end-product of respiratory oxidation and CO_2/HCO_3^- is the body's primary pH buffer system. The bicarbonate transport super-family of genes (SLC4 and SLC26 gene families), responsible for transmembrane movement of membrane-impermeant HCO_3^-, is comprised of the Cl^-/HCO_3^- anion exchanger (AE) family (Alper et al., 1988; Kopito et al., 1989; Kudrycki et al., 1990), the Na^+/HCO_3^- co-transporter proteins (NBC) (Boron & Boulpaep, 1989; Burnham et al., 1997) and the recently identified proteins pendrin (Everett et al., 1999; Scott et al., 1999; Soleimani et al., 2001) and DRA (Melvin et al., 1999; Rajendran et al., 2000; Wheat et al., 2000).

Several lines of evidence have demonstrated an interaction between CAII and the AE1, AE2 and AE3 anion exchanger isoforms. Binding of erythrocyte membranes to CAII increased CAII enzymatic activity (Parkes & Coleman, 1989), which suggested an interaction between these two proteins. CAII can be co-immunoprecipitated with solubilised AE1 and incubation with an extracellular lectin caused agglutination of AE1 and a similar redistribution of CAII on the cytosolic surface of the erythrocyte membrane (Vince & Reithmeier, 1998). A sensitive microtiter binding assay, using truncation and point mutation of the AE1 C-terminus, led to the identification of the binding site of CAII in AE1 as LDADD (amino acids 886-890) (Vince & Reithmeier, 2000) and the basic amino-terminal region of CAII as the binding site for AE1 (Vince et al., 2000).

LDADD (amino acids 886-890) (Vince & Reithmeier, 2000) and the basic amino-terminal region of CAII as the binding site for AE1 (Vince et al., 2000).

The functional consequences of the AE/CAII interaction have been studied (Sterling et al., 2001). Using HEK 293 cells transiently transfected with AE1 cDNA, we determined that inhibition of endogenous CAII activity with acetazolamide resulted in a decrease of AE1 transport activity. Mutation of the AE1, LDADD CAII binding motif caused a loss of CAII binding and a corresponding 90% decrease of AE1 transport activity. Over-expression of the functionally inactive CAII mutant, V143Y (Fierke et al., 1991), displaced wild-type CAII from all three members of the AE family and had a dominant-negative effect on anion transport, inhibiting transport by approximately 50%. This demonstrated that binding of functional CAII to the C-terminus of AE1, AE2 and AE3 proteins is required for maximal bicarbonate transport activity. The requirement of a physical interaction between CAII and AE for maximal bicarbonate transport provided the first direct evidence of a functional transport metabolon (Figure 4.9): a physically associated complex of proteins in a metabolic pathway (Srere, 1985; Srere, 1987). The metabolon may accelerate the coupled production of bicarbonate and transport by minimisation of the diffusional distance between CAII and the bicarbonate transporter. The interaction between CAII and a peptide corresponding to the LDADD motif has also been reported to stimulate CAII activity directly (Scozzafava & Supuran, 2002). Together these effects will increase substrate concentration at the transport site, thereby stimulating bicarbonate transport.

Comparison of the amino acid sequences of the C-terminal tails of bicarbonate transport proteins shows that, with the exception of DRA (down-regulated in adenoma) (Schweinfest et al., 1993), all of these proteins contain at least one consensus CAII binding motif, consisting of a hydrophobic residue followed by four amino acids of which at least two are acidic residues (Vince & Reithmeier, 2000) (Figure 4.7). Formation of a transport metabolon with CAII may therefore also occur with other bicarbonate transport proteins. Na^+/HCO_3^- co-transporters also physically interact with CAII and this interaction, as with the AE family, is necessary for their maximal transport activity (Alvarez, Loiselle and Casey, manuscript in preparation (NBC1) and Loiselle and Casey, manuscript in preparation, (NBC3)). The absence of a potential CAII binding site in DRA therefore raises the questions of whether DRA forms a complex with CAII and if it requires the formation of such a complex to maximise the rate of bicarbonate transport. If DRA does not interact with CAII, it differs significantly from the other bicarbonate transporters, particularly in its mode of regulation.

Human DRA, cloned from a colon subtraction library, is expressed in the normal colon but not in most adenocarcinomas (Schweinfest et al., 1993). The protein product of the DRA gene is a membrane glycoprotein predicted to span the membrane 10-14 times (Byeon et al., 1996). The protein is related to the sulfate transporters DTSDT (Hastbacka et al., 1994) and SAT-1 (Bissig et al., 1994) and has been shown to transport sulfate when expressed in oocytes (Silberg et al., 1995). Although DRA has considerably less similarity to the Cl^-/HCO_3^- anion exchange (AE) protein family, DRA also mediates Cl^-/HCO_3^- exchange activity

when expressed in cultured mammalian cells (Greeley et al., 2001; Melvin et al., 1999).

Cl^-/HCO_3^- exchange function in the human colon and ileum has been attributed to DRA (Melvin et al., 1999; Rajendran et al., 2000), which works in concert with the Na^+/H^+ exchanger (NHE) to mediate NaCl absorption. Mutations in the DRA gene manifest as congenital chloride diarrhea (CLD), an autosomal recessive disorder of intestinal electrolyte absorption (Hoglund et al., 1998; Hoglund et al., 1996). Studies of humans with CLD provide strong evidence for defects in Cl^-/HCO_3^- exchange in the ileum and colon (Holmberg et al., 1975).

Cystic Fibrosis (CF) is an autosomal recessive disease arising from inactivation or mis-processing of a cAMP sensitive Cl^- channel, known as the CF transmembrane conductance regulator (CFTR) (Rosenstein & Zeitlin, 1998). CF causes defective fluid and electrolyte secretion in secretory epithelia (Quinton, 1990; Welsh & Fick, 1987), which impairs the respiratory, pancreatic, hepatobilliary and genitourinary systems (Rosenstein & Zeitlin, 1998). Two recent papers have shown that the expression of DRA in both trachea epithelial cells and cultured pancreatic duct cells increases in the presence of active CFTR. This increase in expression of DRA is accompanied by an increase in Cl^-/HCO_3^- transport activity (Greeley et al., 2001; Wheat et al., 2000). The authors concluded that the HCO_3^- secretion defect in patients with CF results in part from the downregulation of the Cl^-/HCO_3^- exchange activity mediated by DRA.

In the present study we investigated the interaction between DRA and CAII. AE Cl^-/HCO_3^- exchangers and the NBC1 and NBC3 Na^+/HCO_3^- co-

transporters require the formation of a transport metabolon with CAII to maximise their bicarbonate transport activity, as discussed above. Using HEK 293 cells transiently-transfected with DRA cDNA, we determined that inhibition of CAII activity with acetazolamide resulted in a substantial decrease of DRA transport activity, indicating that DRA requires the presence of active CAII for maximal bicarbonate transport. A microtiter plate assay showed that the C-terminal tail of DRA binds CAII with a much lower affinity and capacity than AE1. Expression of functionally inactive V143Y CAII mutant (Fierke et al., 1991) displaced wild-type CAII from AE1 and had a dominant-negative effect on anion transport (Sterling et al., 2001). In contrast, over-expression of V143Y CAII had no effect on the rate of DRA-mediated bicarbonate transport. Taken together these results indicate that although DRA activity requires the presence of CAII enzymatic activity in the cytosol, DRA and CAII do not form the physical complex required by AE and NBC. Therefore, DRA is thus far unique among HCO_3^- transport proteins because it does not form a complex with CAII. This suggests that the regulation of DRA activity differs from NBCs and AEs.

5.2 Results

5.2.1 HCO_3^- transport activity of DRA

To measure the anion exchange transport activity of the protein DRA, HEK 293 cells were transiently transfected with human DRA cDNA. The transfected cells were grown on coverslips and loaded with BCECF-AM, a pH sensitive fluorescent dye. Cells were then perfused alternately with chloride-containing and chloride-free Ringer's buffer. In chloride-free Ringer's buffer, chloride will leave the cell via DRA in exchange for HCO_3^-, causing alkalinisation

of the cytosol. Conversely, in chloride-containing Ringer's buffer, chloride will now move into the cell in exchange for the removal of HCO_3^-, resulting in cellular acidification. Changes in pH_i are measured as changes in fluorescence of BCECF and, following appropriate calibration, this experiment results in an indirect measurement of changes in intracellular pH resulting from DRA-mediated HCO_3^- flux (Figure 5.1). Rates of transport were obtained by linear regression of the initial changes of pH_i following the change of Ringer's buffer. The anion exchange transport rate for cells transiently transected with vector alone was subtracted from the total rate of transport obtained with the cells expressing DRA, to give a transport rate for DRA alone.

DRA transported bicarbonate with a rate of 0.3 ± 0.02 ΔpH/min. Previous determination of the intrinsic buffering capacity of these cells (Lee et al., 1991; Sterling & Casey, 1999) enables the rate of change in pH_i to be converted to H^+ flux. When applied to DRA the ΔpH_i/min rate represents a H^+ equivalent flux of 18 ± 1 mM/min. Thus under similar transient transfection conditions, DRA has anion exchange activity about half of that found for human AE1 in transfected HEK 293 cells (40 ± 1 mM/min, (Sterling et al., 2001)).

5.2.2 Effects of CAII inhibition on DRA Cl^-/HCO_3^- exchange activity

HEK 293 cells endogenously express CAII (Sterling et al., 2001). To determine the contribution of CAII activity to the HCO_3^- transport rate of DRA, we compared the transport rate of DRA-expressing cells before and after incubation with acetazolamide, a sulfonamide that inhibits CA enzymatic activity without direct effect on anion exchange (Cousin & Motais, 1976; Cousin et al., 1975). Transiently-transfected cells were grown on glass coverslips and

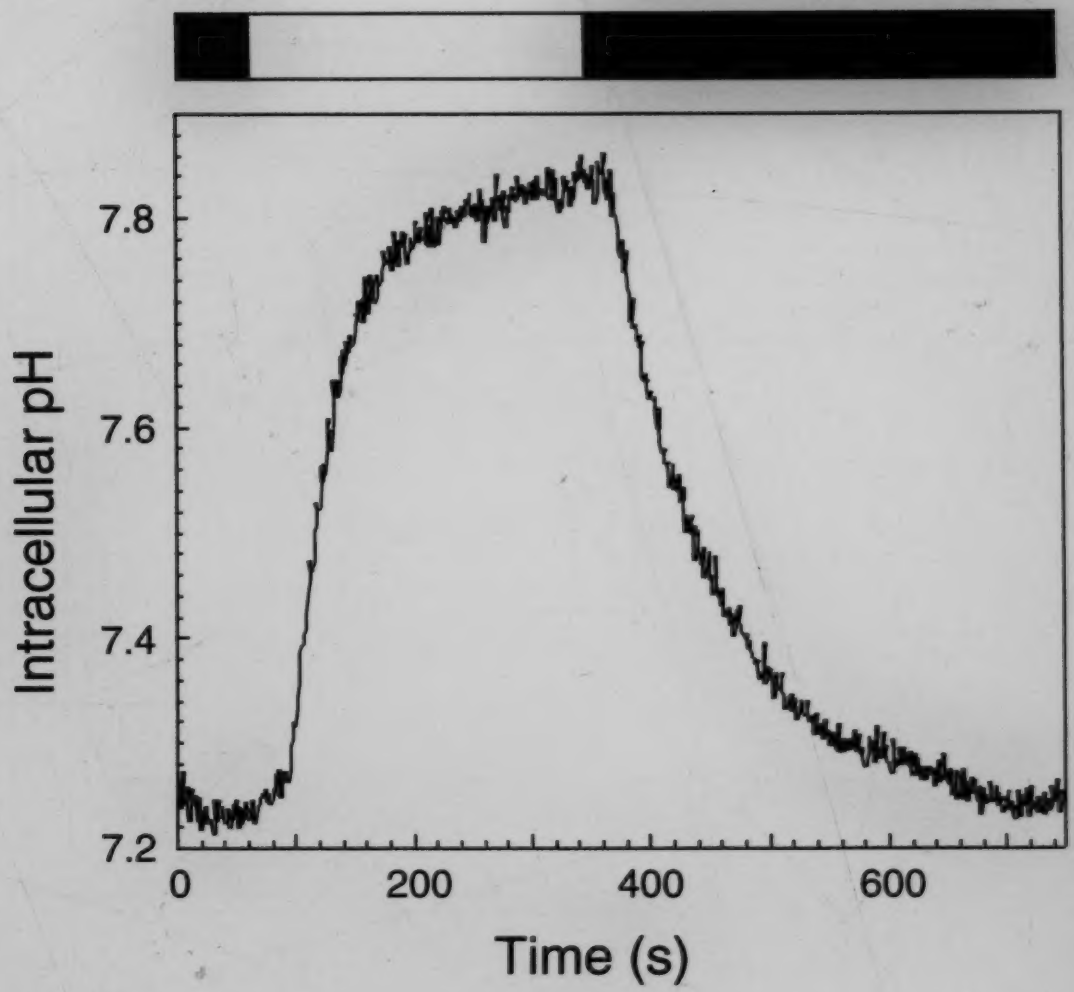

Figure 5.1 Chloride/bicarbonate exchange activity of DRA.
HEK 293 cells transfected with DRA cDNA were loaded with BCECF-AM and placed in a fluorescence cuvette in a fluorimeter. Cells were perfused alternately with Cl^--containing (solid bar) and Cl^--free (open bar) Ringer's buffer and fluorescence was monitored at excitation wavelengths 440 and 502.5 nm and emission wavelength 528.7 nm.

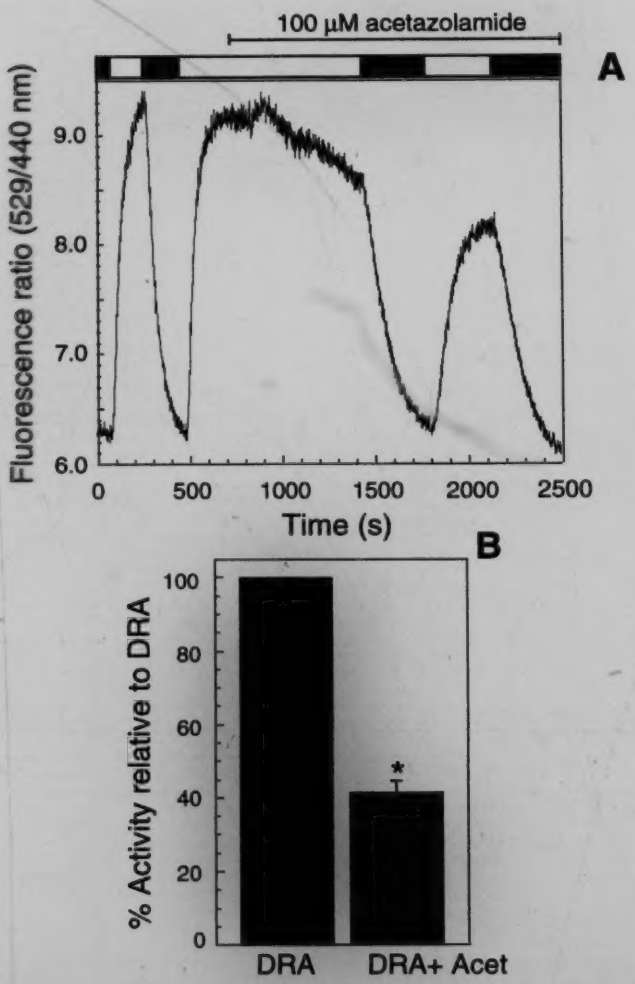

Figure 5.2 Full DRA transport activity requires active carbonic anhydrase. HEK 293 cells transfected with DRA cDNA were loaded with BCECF-AM and subjected to the same transport assay as described in fig. 3. Perfusion with Cl^--containing Ringer's buffer is indicated by the solid bar and perfusion with Cl^--free Ringer's buffer by the open bar. Cells were then incubated with 100 µM acetazolamide for 10 minutes followed by a repeat of the Ringer's buffer perfusion in the presence of 100 µM acetazolamide, as indicated. Transport activity following acetazolamide incubation was compared to that before and expressed as a percentage of this transport activity. A, effect of 100 µM acetazolamide on DRA-mediated HCO_3^- transport. B, relative degree of inhibition of DRA-mediated HCO_3^- efflux following acetazolamide incubation. Standard error bars are indicated (n=4) and asterisk denotes a significant difference in transport rate ($p<0.05$).

subjected to the transport assay described above. Following an initial perfusion with chloride-free and chloride-containing Ringer's buffer, cells were incubated for 10 minutes with 100 μM acetazolamide. As acetazolamide does not covalently react with CA, all buffers used subsequently in the experiment also contained 100 μM acetazolamide. Figure 5.2 shows that treatment with 100 μM acetazolamide caused a 49 ± 5% reduction in DRA-mediated bicarbonate flux, indicating that DRA requires the presence of functional CAII for maximal transport activity.

5.2.3 Binding of CAII to DRA

The amino acid sequences of the cytoplasmic C-terminal regions of HCO_3^- transport proteins all possess a potential CAII binding site (a hydrophobic residue followed by a group of four amino acids, of which at least two must be negatively charged (Vince & Reithmeier, 2000)), except for DRA (Figure 4.7). Although the C-terminal tail of DRA does not contain a potential CAII binding site, we cannot assume that it will not be able to bind to CAII. To investigate the ability of DRA C-terminus to bind CAII we constructed a GST fusion protein of the cytoplasmic C-terminal region of DRA and measured its capacity to bind CAII.

Figure 5.3 shows the results of the microtiter assay, which measured binding between GST fusion proteins (GST-AE1ct and GST-DRAct, respectively) and CAII immobilised on a 96 well plate. Both GST-AE1ct and GST-DRAct bound more CAII than did GST alone. However, AE1 bound CAII much more avidly than did DRA. Binding affinities of interactions were Kd of 60 nM for DRA and 40 nM for AE1. The maximum binding capacity of DRA was only 25%

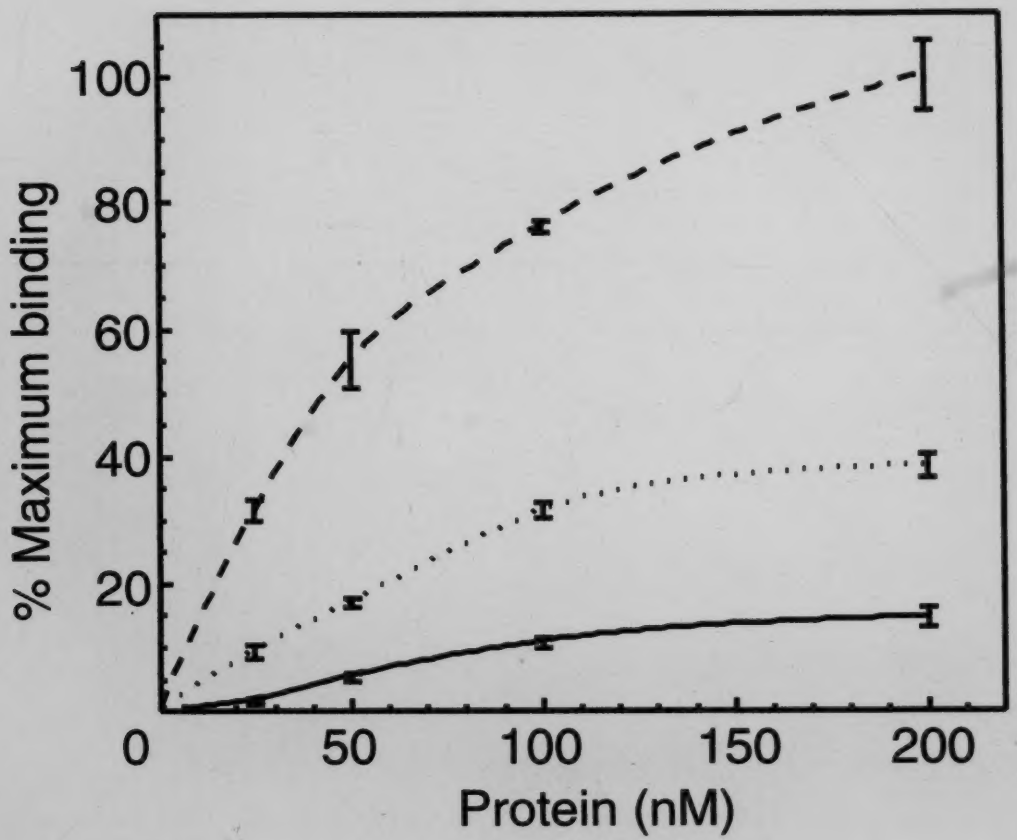

Figure 5.3 CAII weakly binds to the carboxyl-terminal tail of DRA.

Wild-type human CAII was immobilised on 96 well microtiter plates. The immobilised CAII was incubated with different concentrations of GST alone (solid line) GST-DRAct (dotted line) and GST-AE1ct (dashed line). Bound proteins were detected by incubation of plates sequentially with goat anti-GST antibody, biotinylated rabbit anti-goat IgG and then peroxidase-labeled biotin/avidin. This was followed by incubation with substrate o-phenyldiamine and detection of product at 450 nm in a microplate reader. Triplicate measurements for each concentration of protein were made on the same plate. Error bars represent standard error of the mean (n =3).

of AE1 (Figure 5.3). This result indicates that the C-terminal domain of DRA binds CAII to a much lower extent than AE1.

5.2.4 Effect of carbonic anhydrases on DRA Cl^-/HCO_3^- exchange activity

The above experiments suggest that, although DRA requires the presence of functional CAII in the cytosol for transport binding activity, little CAII will bind the DRA C-terminal tail. However, CAII may bind another region of DRA.

To assess the effect of a DRA-CAII physical interaction upon transport activity, we employed a dominant-negative approach, previously used for AE proteins (Sterling et al., 2001). HEK 293 cells transfected with V143Y CAII, a functionally inactive mutant (Fierke et al., 1991), express the mutant CAII at levels about 20 fold higher than endogenous wild-type CAII (Sterling et al., 2001). V143Y CAII, retains its ability to bind to the AE HCO_3^- transport proteins (Sterling et al., 2001), so that over-expression of V143Y CAII will compete with the endogenous wild-type CAII at any potential binding site in the cell. Thus if DRA is activated by a physical interaction with CAII, as are other HCO_3^- transport proteins, over-expression of V143Y CAII will decrease the transport activity of DRA.

HEK 293 cells were co-transfected with CAII and DRA cDNAs. Figure 5.4 shows that over-expression of either wild-type CAII protein or V143Y CAII had no effect on DRA transport activity. The lack of effect of over-expression of wild-type CAII suggests that the endogenous level of CAII is sufficient for full DRA transport activity, consistent with results for the AE Cl^-/HCO_3^- exchangers (Sterling et al., 2001). Since V143Y CAII did not alter DRA transport activity,

DRA does not require a direct physical association with CAII for maximal transport activity.

We have found recently that the human AE1 Cl^-/HCO_3^- anion exchanger also physically interacts with membrane-anchored CAIV, acting as the extracellular component of the bicarbonate transport metabolon (Sterling, Alvarez and Casey, submitted for publication). The loss of Cl^-/HCO_3^- anion exchange activity of AE1 caused by V143Y CAII could be rescued by expression of CAIV. However, figure 5.4 shows that co-expression of CAIV did not have any effect on DRA activity. We conclude that, unlike AE1, DRA does not functionally interact with CAIV.

5.3 Discussion

In this study, we have examined the physical and functional relationships between DRA and CAII. The lack of a CAII binding site motif in the C-terminal tail of DRA thus far makes DRA a unique bicarbonate transport protein. To investigate whether CAII activity had any impact on the transport capability of DRA we monitored DRA-mediated bicarbonate transport activity before and after incubation with 100 μM acetazolamide, a membrane permeant CA inhibitor (Cousin & Motais, 1976; Cousin et al., 1975). Our data showed that inhibition of CAII by acetazolamide impaired DRA transport activity, which indicates that CAII activity has a substantial effect on DRA Cl^-/HCO_3^- exchange activity. Thus, DRA bicarbonate transport activity and CAII activity are functionally coupled. However, binding assays showed that the ability of the DRA C-terminal tail to bind CAII is much less than the bicarbonate transporter, AE1. Since the binding assay showed some limited interaction between CAII and DRA, it was possible

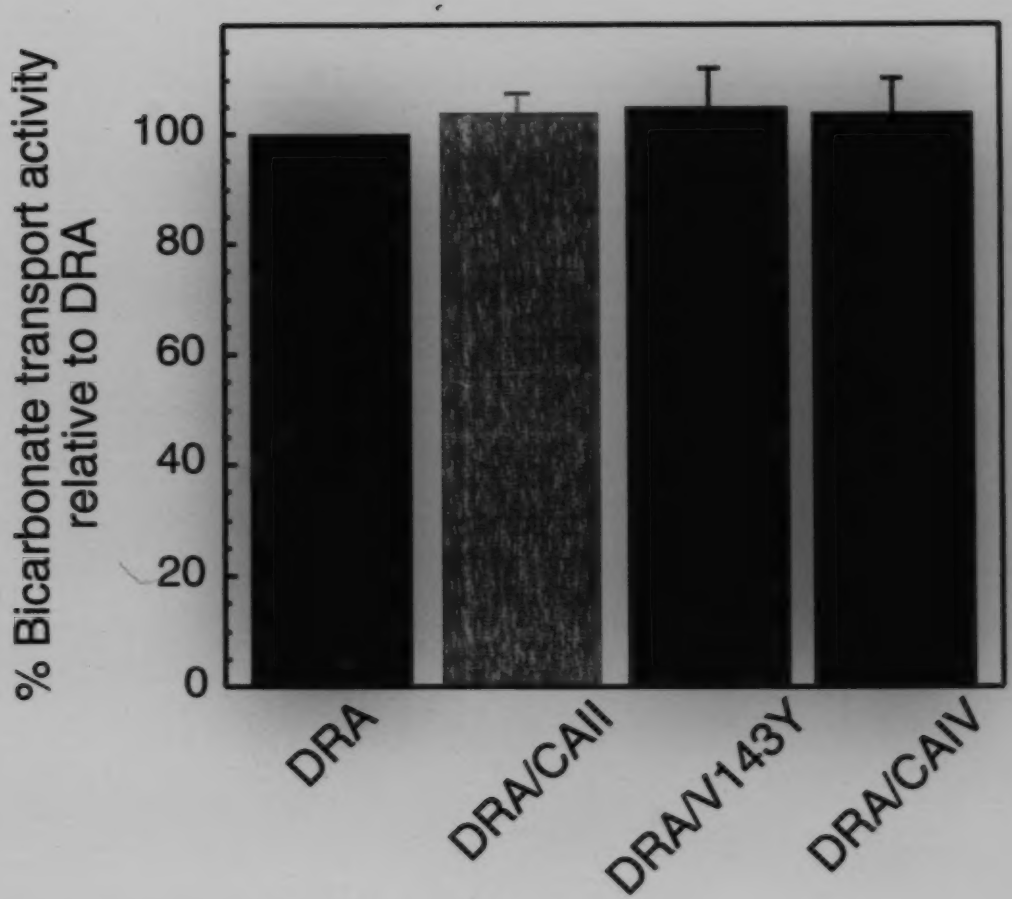

Figure 5.4 Effect of expression of functionally inactive V143Y CAII on DRA transport activity.

HEK 293 cells grown on coverslips were transiently co-transfected with cDNA encoding DRA and a combination of wild-type CAII, V143Y CAII, or CAIV, as indicated. Two days post transfection cells were subjected to anion exchange assays. Transport rates are expressed relative to the rate of DRA expressed alone. Standard error bars are indicated (n=3-6). No statistically significant difference between values was found.

that interaction with CAII activates DRA. However, over-expression of the V143Y CAII mutant had no dominant-negative effect on DRA transport activity. This indicated that DRA bicarbonate transport activity is not activated by CAII binding at any site on DRA. We conclude that DRA may bind to CAII to some limited degree, but that any physical interaction between the two proteins has no impact on the bicarbonate transport activity of DRA.

The bicarbonate transport metabolon is emerging as an important mechanism to accelerate and regulate bicarbonate transport activity. A transport metabolon is a physical complex of an enzyme and a transporter, which maximises and may regulate substrate flux through the enzyme and across the membrane. The first example of a bicarbonate transport metabolon was provided by anion exchangers of the AE family, which form a functional and physical complex with cytosolic CAII (Kifor et al., 1993; Parkes & Coleman, 1989; Vince & Reithmeier, 1998) and with the extracellular enzyme, CAIV (Sterling, Alvarez and Casey, submitted for publication). NBC1 and NBC3 also form a metabolon with CAII (Alvarez, Loiselle and Casey, manuscript in preparation (NBC1) and Loiselle and Casey, manuscript in preparation (NBC3)). Comparison of the C-terminal tails of other bicarbonate transport proteins indicates that, with the exception of DRA, they all contain at least one potential CAII binding site (Figure 4.7). The analysis performed in this report indicates that, consistent with the absence of a CAII binding motif, binding of CAII or CAIV had no effect on DRA transport activity.

When DRA was first cloned from mouse (Melvin et al., 1999), the Cl^-/HCO_3^- transport activity of the protein was also characterised. The

investigators monitored transport activity in the absence of HCO_3^- and found that DRA mediated a slow alkalinisation upon removal of extracellular chloride. Cells did not recover from the alkalinisation upon reintroduction of extracellular Cl^-, which was attributed to a high specificity at the intracellular substrate site for HCO_3^- (Melvin et al., 1999). The fact that DRA does not form a transport metabolon with CAII provides another explanation for the lack of recovery of the intracellular pH to a physiological level. In the absence of extracellular bicarbonate, re-addition of extracellular chloride will cause chloride to move into the cell in exchange for bicarbonate. We have shown that tethering of CAII near the intracellular anion-binding site of AE1 increases the rate of HCO_3^- efflux. Since DRA does not bind CAII, the production of intracellular HCO_3^- will occur in the cytosol as opposed to at the site of anion exchange, thus slowing the efflux of HCO_3^- and thus decreasing the rate of recovery of intracellular pH.

A decreased transport rate due to a failure to interact with CAII is also consistent with the observation that the transport activity of DRA is approximately half of that seen for AE1, when expressed in HEK 293 cells. The fact that DRA binds CAII to a much lesser degree that AE1 may provide a reason for this. Over-expression of the dominant-negative V143Y CAII reduced AE1 transport activity by 60% (Sterling et al., 2001), reducing it to an activity comparable to that of DRA. Therefore, DRA may not function at its maximum capacity because it does not form a stoichiometric complex with CAII.

The data presented here shows that DRA is thus far unique among bicarbonate transporters because it does not interact with either CAII or CAIV. One reason for this may be that DRA does not function solely as a Cl^-/HCO_3^-

transport protein, consistent with reports that DRA transports sulfate, oxalate and chloride (Moseley et al., 1999; Silberg et al., 1995). Similarly, while investigating Cl^-/HCO_3^- exchange in apical membrane vesicles from human proximal colon, it was noted that bromide, nitrate and acetate inhibited uptake of $^{36}Cl^-$, suggesting that the colonic Cl^-/HCO_3^- exchange protein, DRA, might also accept these ions as substrates (Mahajan et al., 1996). However, there is strong evidence that the physiological role of DRA in the mammalian colon is Cl^-/HCO_3^- exchange (Melvin et al., 1999).

The lack of interaction between DRA and carbonic anhydrases also suggests that DRA is regulated differently than other bicarbonate transporters. Direct interaction between carbonic anhydrase and a bicarbonate transporter accelerates the bicarbonate transport rate (Sterling et al., 2001). Modulation of the interaction therefore presents a rapid way to alter the rate of bicarbonate transport, but DRA cannot use this mode of regulation. In the physiological context, cells may contain a membrane protein that binds CAII and localises the enzyme near the DRA transport site. If such a protein exists, HEK 293 cells do not express it since dominant negative V143Y CAII had no effect on DRA activity.

DRA is expressed on the apical membrane of pancreatic epithelia (Greeley et al., 2001). In response to the release of secretin, the pancreas produces a HCO_3^--rich alkaline fluid, which is secreted to the duodenum to neutralise the acidic chyme produced during digestion. Bicarbonate uptake across the basolateral membrane of pancreatic cells occurs via an NBC (Shumaker et al., 1999), whereas bicarbonate flux across the apical membrane may be mediated by

DRA (Greeley et al., 2001). Cystic fibrosis patients frequently present with a greatly reduced bicarbonate secretion capacity (Quinton, 1990). It has been shown recently that expression of DRA is increased in the presence of functional CFTR in both cultured pancreatic and tracheal cells (Greeley et al., 2001; Wheat et al., 2000). The increase in DRA expression in pancreatic cells was associated with a 2 fold increase in Cl^-/HCO_3^- transport activity and the authors concluded that the decrease in HCO_3^- production in CF patients is in part due to downregulation of DRA expression and activity. Our data is consistent with a model in which defective pancreatic bicarbonate secretion in CF results from the inability of DRA to bind directly to CAII. Failure to localise CAII close to the DRA bicarbonate transport site could reduce transport efficiency. An intermediary protein may be required to bring CAII to the vicinity of DRA for optimal DRA bicarbonate transport. This intermediary may be CFTR or some protein regulated by CFTR.

With the exception of DRA, the C-terminal tail of every bicarbonate transport protein contains a potential CAII binding motif (Figure 4.7). Formation of a complex with CAII potentiates bicarbonate transport activity and modulation of the interaction provides a cell with a potential regulatory mechanism to control bicarbonate flux (Sterling et al., 2001). DRA is the only bicarbonate transport protein identified to date in which the CAII binding site is absent. In this study, we investigated the physical and functional interaction between DRA and CAII. Based on our results we conclude that although there is a need for the presence of CAII enzymatic activity in the cytosol, the weak physical interaction between the two proteins does not affect the functional

activity of DRA. For full transport activity DRA may require an intermediary protein to bind CAII and bring the enzyme close to DRA in the plasma membrane. This makes DRA unique among the bicarbonate transport protein superfamily, examined to date.

Chapter 6

The extracellular component of a transport metabolon: Extracellular loop 4 of the human AE1 Cl^-/HCO_3^- exchanger binds carbonic anhydrase IV[5]

[5]Portions of this chapter have been accepted for publication and previously published in abstract form:

Sterling, D., Alvarez, B. V. and Casey, J.R. (2002) The extracellular component of a transport metabolon: Extracellular loop 4 of the human AE1 Cl^-/HCO_3^- exchanger binds carbonic anhydrase IV, *J. Biol. Chem.* In press

Sterling, D., Alvarez, B. V. and Casey, J.R. (2002) Carbonic anhydrase IV potentiates the bicarbonate transport activity of Cl^-/HCO_3^- anion exchange proteins, *Biophysical Journal*, **82**, 570

The extracellular component of a transport metabolon: Extracellular loop 4 of the human AE1 Cl^-/HCO_3^- exchanger binds carbonic anhydrase IV

6.1 Introduction

Carbonic anhydrases (CA) are a family of zinc metalloenzymes (EC 4.2.1.1) that catalyze the rapid hydration/dehydration of CO_2/HCO_3^-. Bicarbonate transport proteins are closely associated functionally with CA and together they eliminate the metabolic waste, CO_2, from the body. There are 14 mammalian isoforms of CA identified to date, varying in catalytic activity and tissue distribution (Kivela et al., 2000a; Mori et al., 1999; Tureci et al., 1998). CAII, found predominantly in red blood cells, has been shown not only to bind to proteins of the AE family of Cl^-/HCO_3^- anion exchange proteins, but also to potentiate their transport activity by formation of a transport metabolon (Sterling et al., 2001; Vince et al., 2000; Vince & Reithmeier, 1998; Vince & Reithmeier, 2000). A metabolon is a complex of proteins involved in a metabolic pathway that allows metabolites to move rapidly from one active site to the next (Srere, 1985; Srere, 1987). The physical association of CAII with AE localises the site of substrate (HCO_3^-) production to the transport site, thus creating a transport metabolon.

The AE family of proteins is comprised of AE1, AE2, and AE3 (Alper et al., 1988; Grinstein, Ship & Rothstein, 1979; Kopito et al., 1989; Kudrycki et al., 1990). The recently cloned AE4, although termed AE, shares little similarity with the other members of the AE family and is in fact more similar to the sodium/bicarbonate co-transporters (NBC) (Tsuganezawa et al., 2001). AE1 is expressed abundantly in erythrocytes and a truncated form is also present in the

kidney and heart (Kollert-Jons et al., 1993; Richards et al., 1999). AE2 is almost ubiquitous, while AE3 expression is restricted to the brain, heart and retina (Alper et al., 1988; Kobayashi et al., 1994; Kopito et al., 1989; Linn et al., 1992).

Unlike cytosolic CAII, CAIV is anchored to the extracellular surface of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, thus reversibly hydrating CO_2 in the extracellular space (Waheed et al., 1992) (Figure 1.10). Northern blots, immunoblots and immunohistochemical analysis, along with functional studies have localised CAIV expression to the heart, lung, kidney, brain, retina and erythrocyte (Brion et al., 1994; Ghandour et al., 1992; Hageman et al., 1991; McKinley & Whitney, 1976; Sender et al., 1998; Sly et al., 1985; Tong et al., 2000; Whitney & Briggle, 1982; Wistrand et al., 1999; Wistrand & Knuuttila, 1989), all of which express AE proteins. CAIV hydrates CO_2 with a catalytic activity of $8x10^5$ sec^{-1}, which is comparable to CAII ($>10^6 s^{-1}$) (Baird et al., 1997). The two CA isoforms differ in their susceptibility to sulphonamide inhibitors, such as acetazolamide, ethoxzolamide, sulphanilamide and benzenesulphonamide, with CAIV having an affinity of 3 - 65 fold less than CAII (Baird et al., 1997). Furthermore, CAIV is unique in that it contains two disulfide bonds that contribute to its stability in 5% SDS, a concentration of denaturant that inactivates CAII (Okuyama et al., 1992).

The wide tissue distribution of AE proteins is mirrored by the broad expression of CA isoforms throughout the body. While some tissues express only one CA isoform, other tissues express multiple isoforms. The extracellular CAIV isoform is expressed in the heart, but there is no evidence for cytosolic CAII (Geers et al., 1992; Sender et al., 1998). Human erythrocytes express CAI,

CAII and CAIV (Vince & Reithmeier, 1998; Wistrand et al., 1999). The kidney, which avidly reabsorbs up to 500 g of $NaHCO_3$/day, expresses both membrane bound CAIV, and cytosolic CAII (Brion et al., 1994; McKinley & Whitney, 1976; Sly et al., 1985; Wistrand & Knuuttila, 1989). CAII localises to the cytosol of cells of renal tubules and collecting ducts, where it is important for the acidification of urine (Sly et al., 1985), while membrane bound CAIV localises to the apical surface of cortical collecting ducts and α-intercalated cells (Schwartz et al., 2000). CAIV plays a major role in bicarbonate reabsorption by the kidney (Maren & Conroy, 1993) as well as modulating the pH in the tubule lumen (Brechue et al., 1991). CAIV is also found on the surface of pulmonary endothelial cells (Whitney & Briggle, 1982) and in the endothelial cells of an ocular capillary bed, where its presence suggests it may be the target for CA inhibitors that are used in the treatment of glaucoma (Hageman et al., 1991). Despite general knowledge of co-localisation of carbonic anhydrase and bicarbonate transporters, the precise structural interrelationships have remained largely unknown. The physiological importance of bicarbonate metabolism and transport led us to investigate the physical and functional relationship between AE proteins and CAIV.

In this study, we found a functional interaction between AE proteins and CAIV. Expression of CAIV had no effect on the bicarbonate transport rate in cells expressing AE1 and cytosolic CAII, since CAII maximises the bicarbonate flux under these conditions. It was not possible to use inhibitors to block CAII function since any membrane-permeable CA inhibitor would access both extracellular CAIV and intracellular CAII. Thus, we used a dominant-negative form of CAII to selectively neutralise the stimulatory effect of cytosolic CAII on

AE transport activity and thereby examine the role of CAIV in AE-mediated bicarbonate transport activity. We found that like CAII, CAIV also accelerates AE-mediated bicarbonate transport activity. Based on co-migration on sucrose gradients, overlay assays and GST pull-down assays we conclude that there is a physical association between extracellular CAIV and the integral membrane transport protein, AE1. The interaction occurs on the fourth extracellular loop of AE1. Taken together, CAIV and AE functionally and physically interact to form the extracellular component of a bicarbonate transport metabolon, which potentiates AE-mediated bicarbonate transport.

6.2 Results

6.2.1 Expression of AE1 and CA in HEK 293 cells

For functional assays, proteins were expressed in HEK 293 cells. This cell line expresses endogenous CAII (Sterling et al., 2001), yet undetectable levels of AE protein (Kopito et al., 1989). All cDNAs were inserted into either the pcDNA3.1 or pRBG4 (Lee et al., 1991) vector, which place them under the control of the cytomegalovirus early gene promoter. Cells were transiently co-transfected with cDNAs encoding AE1, the functionally inactive mutant V143Y CAII, and CAIV. Figure 6.1 indicates that transient co-transfection of HEK 293 cells with cDNAs encoding AE1, CAII and CAIV results in expression of all three proteins. Cells transfected with vector alone showed no immunoreactivity with AE or CAIV antibodies but did indicate the presence of endogenous CAII at a level approximately 20-fold lower than in CAII-transfected cells (not shown). CAIV on immunoblots frequently appeared as two bands. The source of this

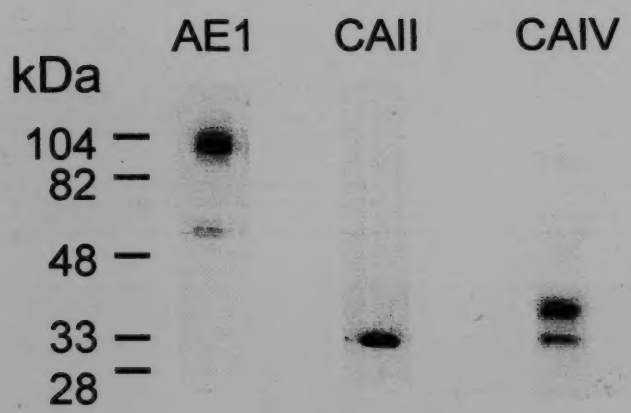

Figure 6.1 Expression of AE1, CAII and CAIV in transfected cells.

HEK 293 cells were transiently co-transfected with cDNA coding for AE1, CAII and CAIV. Two days post transfection, cells were solubilised. Samples (5 µg protein) were resolved by SDS-PAGE on 8% (samples probed for AE1) or 12.5 % (samples probed for CAII and CAIV) acrylamide gels and transferred to PVDF membrane. Immunoblots were probed with rabbit polyclonal antibody, 1658, directed against the C-terminus of human AE1, sheep-anti human CAII antibody or goat anti-rabbit CAIV as indicated.

doublet is not clear but the difference in size is consistent with either two different glycosylated forms (Schwartz et al., 2000), or from partial protein processing, leaving an uncleaved transmembrane anchor on the protein.

6.2.2 Cl^-/HCO_3^- exchange activity

To measure anion exchange activity, transiently transfected cells were grown on coverslips and loaded with BCECF-AM, a pH sensitive fluorescent dye. The coverslips were placed in a fluorescence cuvette and perfused alternately with chloride-containing and chloride-free Ringer's buffer. In chloride-free Ringer's buffer, chloride leaves the cell and bicarbonate enters resulting in cell alkalinisation. In chloride-containing Ringer's buffer, the opposite happens, with chloride entering the cell in exchange for bicarbonate, leading to cell acidification. Following appropriate calibration using the high potassium nigericin technique (Thomas et al., 1979), changes in fluorescence of BCECF provide an indirect measure of changes in intracellular pH associated with chloride bicarbonate exchange activity.

To determine the effect of CAIV on AE transport activity we co-transfected HEK 293 cells individually with AE1, AE2 or AE3c and CAIV cDNAs. Co-expression of AE proteins with CAIV had no effect on the AE-mediated bicarbonate transport activity (data not shown). An effect of CAIV may not have been evident because HEK 293 cells endogenously express sufficient CAII to maximise AE transport activity (Sterling et al., 2001). To separate any effect CAIV might have on AE transport activity from that of CAII, we over-expressed a functionally inactive V143Y CAII mutant (Fierke et al., 1991). Transfection of HEK 293 cells with V143Y CAII resulted in expression 20

fold over endogenous CAII levels (not shown). V143Y CAII acts in a dominant-negative manner to displace functional wild-type CAII from cellular binding sites, thus reducing AE transport activity by blockage of the functional AE/CAII metabolon (Sterling et al., 2001).

Figure 6.2 shows that expression of V143Y CAII substantially reduced AE1 transport activity (53 ± 3% inhibition). Strikingly, addition of CAIV to AE1 and V143Y CAII fully rescued transport activity of AE1, restoring the bicarbonate transport rate to the same level as cells expressing AE1 and wild-type CAII alone (Figure 6.2). The CAIV-induced rescue of AE1 transport activity indicates a functional interaction between AE1 and CAIV. This result implies that the AE1 bicarbonate transport rate can be maximised by an interaction with either CAII or CAIV. To determine whether the rescue of AE1 transport activity by CAIV was dependent on CAIV catalytic activity, we compared the transport activity of cells expressing AE1, V143Y CAII and CAIV before and after incubation with the CA inhibitor acetazolamide (Figure 6.2). Acetazolamide is a membrane-permeant inhibitor of both CAII and CAIV that has no direct effect on anion exchange activity (Cousin & Motais, 1976; Cousin et al., 1975). The presence of 100 μM acetazolamide abolished the CAIV-induced rescue of AE1 transport activity (50 ± 1% inhibition) (Figure 6.2D). This indicates that the rescue of AE1 transport activity by CAIV was dependent on the catalytic activity of CAIV. The co-expression of V143Y CAII also reduced transport activity of AE2 and AE3c (49 ± 10% and 35 ± 1% inhibition respectively) (Figure 6.3). Figure 6.3 also demonstrates that co-expression of CAIV with V143Y CAII

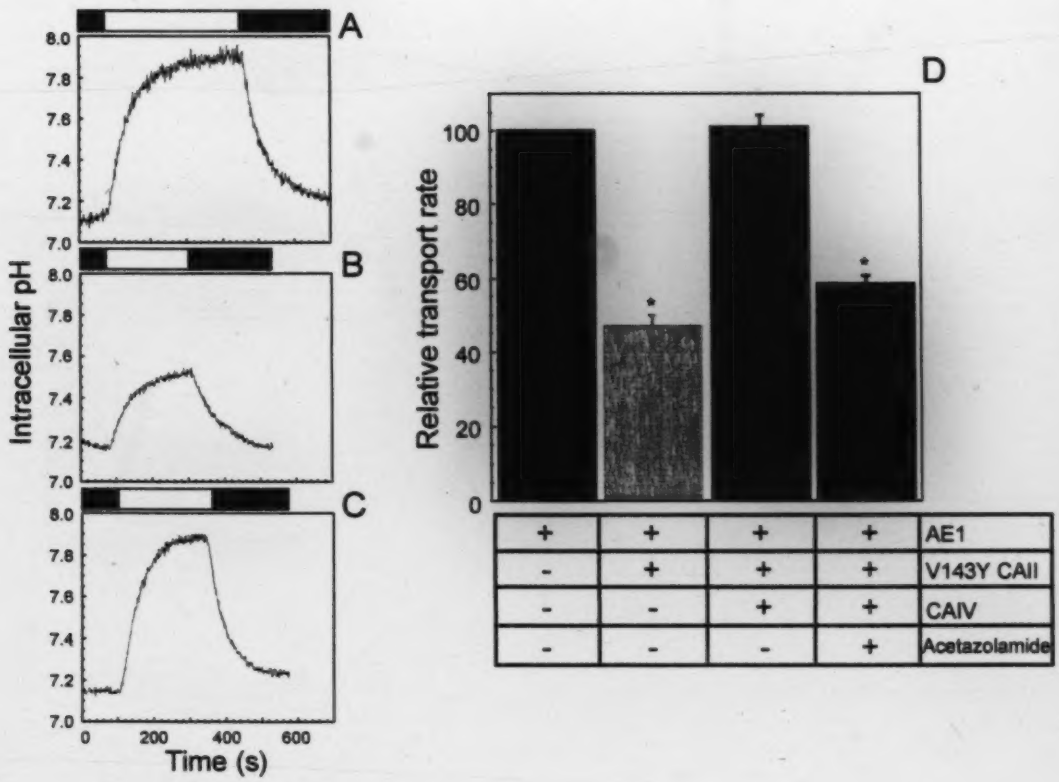

Figure 6.2 Effect of carbonic anhydrases on AE1 transport activity.

HEK 293 cells grown on coverslips were transiently co-transfected with cDNA encoding AE1 (A), AE1 and V143Y CAII (B) and AE1, V143Y CAII and CAIV (C). Two days post transfection cells were loaded with BCECF-AM and placed in a fluorescence cuvette in a fluorimeter. Cells were perfused alternately with Cl^--containing (solid bar) and Cl^--free (open bar) Ringer's buffer and fluorescence was monitored using excitation wavelengths 440 and 502.5 nm and emission wavelength 528.7 nm. In some experiments, cells were incubated with 100 µM acetazolamide for 10 minutes followed by a repeat of the Ringer's buffer perfusion in the presence of 100 µM acetazolamide. Transport activity following acetazolamide incubation was compared to that before the incubation. D, summary of transport rates expressed relative to rate for AE1 expressed alone. Error bars represent standard error of the mean (n =4) and asterisk represents statistical significance ($p<0.001$).

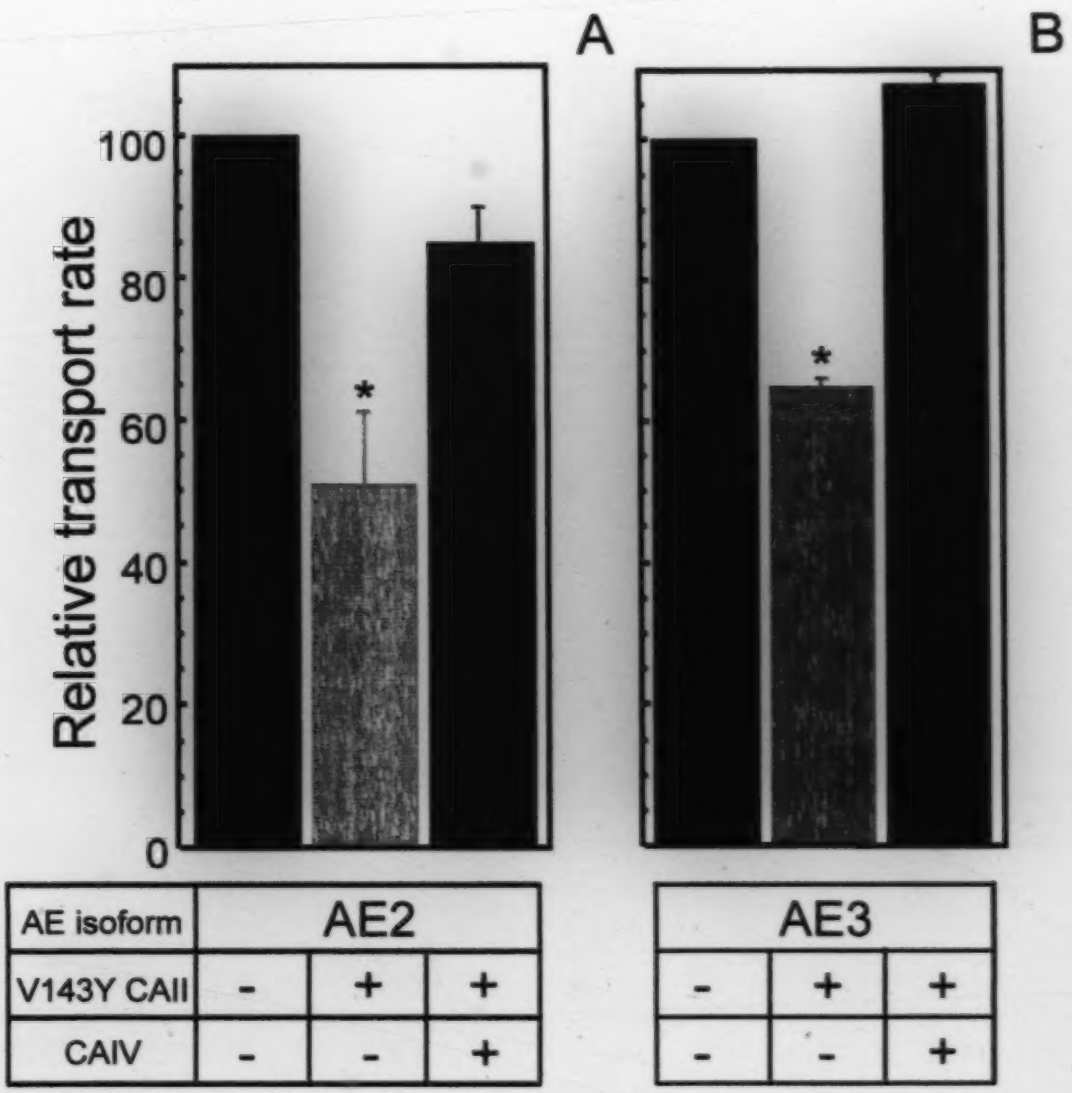

Figure 6.3 CAIV facilitates bicarbonate transport by AE2 and AE3.
HEK 293 cells were transiently transfected with cDNA encoding AE2 or AE3 and co-transfected with or without V143Y CAII and CAIV cDNA, as indicated at the bottom of the figure. Anion exchange activity was measured and rates expressed relative to the rate for AE2 (panel A) and AE3 (panel B). Error bars represent standard error of the mean (n =4) and asterisk represents statistical significance ($p<0.001$).

rescued AE2 and AE3c transport activity to full capacity (85 ± 5% and 108 ± 1% respectively), which indicates a functional interaction with CAIV.

6.2.3 Sucrose Density Ultracentrifugation

CAIV resides on the extracellular surface of cells, anchored via a GPI linkage (Waheed et al., 1992). GPI-anchored proteins localise to lipid rafts in the plasma membrane (Harder & Simons, 1997) and it can therefore be assumed that CAIV, being GPI-anchored, also localises to lipid rafts. Cold solubilisation of membranes with Triton X-100 leaves lipid rafts intact while solubilising the rest of the membrane (Brown & Rose, 1992). Subsequent sucrose density centrifugation allows separation of proteins according to density. We used this technique to investigate the possibility of a physical interaction between CAIV and AE1. HEK 293 cells transiently transfected with either AE1 or CAIV or with both AE1 and CAIV were selectively solubilised and samples overlaid onto 5-30% continuous sucrose gradients. Following 16-24 h ultracentrifugation, fractions were collected and the relative amount of AE1 and CAIV in each fraction was measured. Figure 6.4 shows that when expressed alone, CAIV is found predominantly in fractions 3 and 4, but when AE1 is expressed alone, AE1 is found predominantly in fraction 7. However, when AE1 and CAIV are co-expressed, AE1 remains predominantly in fractions 7/8 while the CAIV shifts to fraction 7. The AE1-dependant shift of CAIV suggests a physical interaction between AE1 and CAIV.

6.2.4 CAIV overlay assay

The interaction between CAIV and AE was further investigated with a blot overlay assay. Cell lysates of HEK 293 cells expressing one of AE1, AE2 or

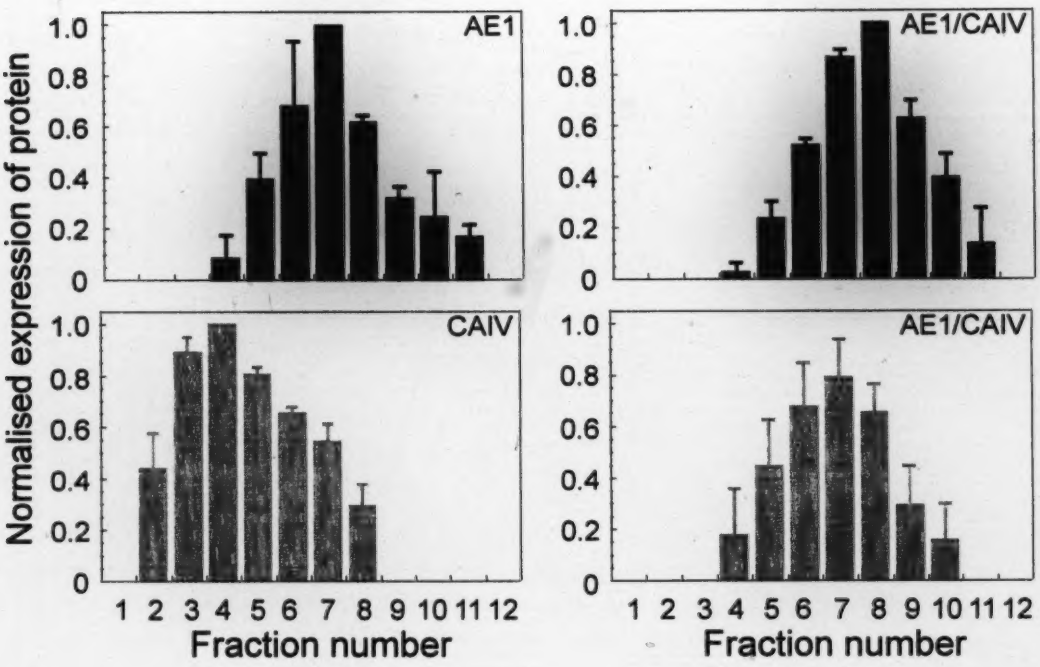

Figure 6.4 Association of AE1 and CAIV.

HEK 293 cells were transiently transfected with cDNA encoding either AE1 or CAIV or co-transfected with both cDNAs, as indicated in each panel. Two days post-transfection cells were solubilised in cold Triton X-100 and samples were overlaid on 5-30% continuous sucrose gradients. Following ultracentrifugation, 12 fractions were collected (1-top, 12-bottom) and samples of each were resolved by SDS-PAGE electrophoresis on an 8% (AE1) or 12.5% (CAIV) polyacrylamide gels. Immunoblotted proteins were probed with either anti-AE1 (black bars) or anti-CAIV (grey bars). Scanning and densitometry of immunoblots provided relative expression levels of protein in each fraction. Error bars represent standard error of the mean (n =3).

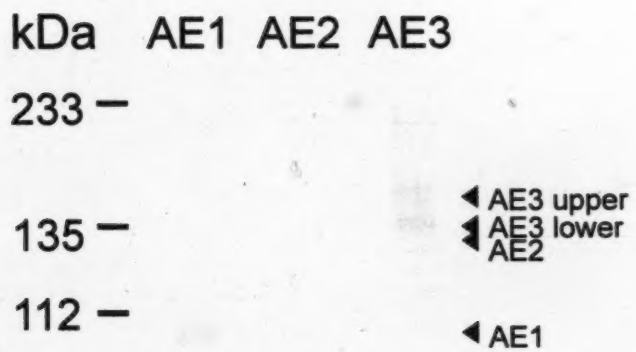

Figure 6.5 Blot overlay assay of CAIV on AE1, AE2 and AE3.

HEK 293 cells were transiently transfected individually with AE1, AE2 or AE3 cDNA. Two days post transfection, cells were solubilised, and 5 µg protein resolved by SDS-PAGE on 8% acrylamide gels and transferred to PVDF membrane, as indicated in the figure. Immunoblots were blocked for 3 hours with 10% TBST-M and then incubated overnight in 1% TBST-M containing a lysate of CAIV-transfected HEK 293 cells. Blots were then probed with anti-CAIV antibody. Arrows indicate position of AE proteins.

AE3c were resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated overnight with a cell lysate of HEK 293 cells, expressing CAIV. Immunoblots were then probed with anti-CAIV antibody. Figure 6.5 shows that CAIV was present at positions corresponding to the migration positions of the AE proteins. No bands were observed in samples from untransfected HEK 293 cells and there are no immunoreactive bands common to all lanes. Thus the bands observed represent a specific interaction of CAIV with only the AE protein present in each lane. This data suggests that there is a physical interaction between CAIV and the AE1, AE2 and AE3 anion exchange proteins.

6.2.5 GST pull-down assays of AE1 extracellular loops

To localise the site of AE1 interaction with CAIV, we reasoned that GPI-anchored CAIV must interact with the extracellular portion of AE1. The most likely candidates for a CAIV binding site are the largest extracellular loops of AE1, EC3 and EC4 (Figure 6.6). GST fusion proteins of the third and fourth extracellular loops of AE1 were used in a GST pull-down assay. GST alone, GST-AE1EC3 and GST-AE1EC4 were immobilised on glutathione Sepharose resin and cell lysates of either sham-transfected or CAIV-transfected cells were applied. After washing, proteins were eluted in SDS-PAGE sample buffer resolved on 8% polyacrylamide gels by SDS-PAGE electrophoresis, transferred to PVDF membranes and probed for CAIV. In figure 6.7 bands were evident only in samples incubated with CAIV-containing lysates. Thus, all bands observed result from CAIV, either full length or possibly shorter proteolytic fragments. GST alone pulled down a small amount of CAIV, as did GST

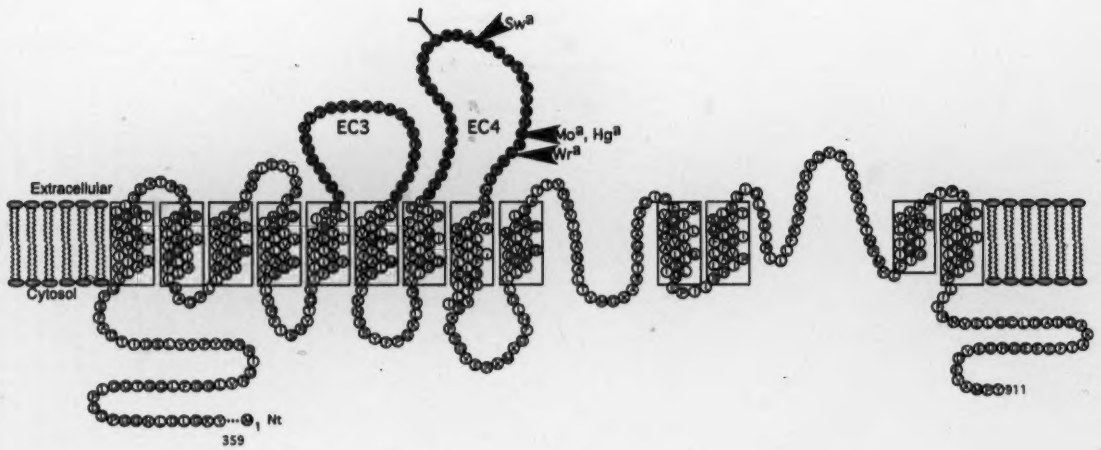

Figure 6.6 Topology model of human AE1.

This model was created on the basis of experimental evidence (Reithmeier et al., 1996; Tang et al., 1998). Amino acids corresponding to GST-AE1EC3 and EC4 are indicated in grey. Arrows indicate the positions of point mutations that induce blood group antigens (Sw^a (R646Q), Mo^a, (R656H), Hg^a (R656C) (Jarolim et al., 1998) and Wr^a (E658K) (Bruce et al., 1995)) are indicated. The Y structure on EC4 marks the position of N-linked glycosylation.

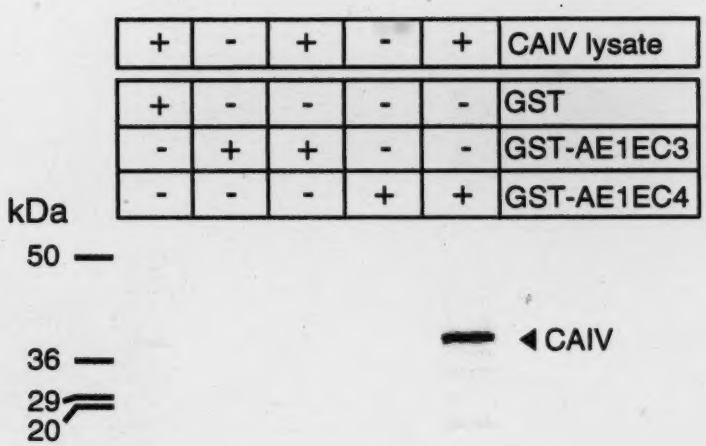

Figure 6.7 CAIV binds specifically to the fourth extracellular loop of AE.
Proteins (10 μg GST alone, GST-AE1EC3 or GST-AE1EC4) were individually bound to glutathione Sepharose resin as indicated. Cell lysates of HEK 293 cells transfected with CAIV cDNA or sham-transfected with vector alone were applied to the beads as indicated and incubated overnight. Samples were centrifuged and the beads washed. Proteins eluted with SDS-PAGE sample buffer were resolved by SDS-PAGE electrophoresis on a 12.5% polyacrylamide gel, transferred to a PVDF membrane and probed for CAIV as described previously. Arrow indicates position of CAIV.

AE1EC3 (Figure 6.7). Clearly, GST-AE1EC4 brought down the most CAIV (Figure 6.7). Indeed, densitometry revealed that GST-AE1EC4 pulled down approximately ten fold more CAIV than did GST alone or GST-AE1EC3. This demonstrates that CAIV binds specifically to the fourth extracellular loop of AE1.

6.3 Discussion

The data presented here show that the expression of CAIV accelerates the rate of bicarbonate transport by AE1, AE2 and AE3. HEK 293 cells endogenously express CAII at a level that is sufficient to maximise the bicarbonate transport activity of the AE family (Sterling et al., 2001). The effect of CAIV on AE transport was found only in the presence of V143Y CAII, which displaces endogenous CAII from its binding site in AE, greatly reducing the anion transport rate (Sterling et al., 2001). While bicarbonate transport by AE1, AE2 and AE3 was inhibited by 35-53% by V143Y CAII, the loss of activity was fully rescued by expression of CAIV. The rescue of AE activity by CAIV was blocked by acetazolamide, a CA inhibitor, indicating that the catalytic activity of CAIV was responsible for the rescue of AE mediated bicarbonate transport activity.

Carbonic anhydrases and bicarbonate transport proteins are together responsible for bicarbonate metabolism and transmembrane transport. Previous studies showed that these proteins form a complex (Kifor et al., 1993; Parkes & Coleman, 1989; Vince et al., 2000; Vince & Reithmeier, 1998; Vince & Reithmeier, 2000) and we have recently provided evidence that the physical interaction between the AE family of bicarbonate transport proteins and CAII is necessary

for maximal HCO_3^- transport activity (Sterling et al., 2001). The wide tissue distribution of CA isoforms raises the question of the possibility of the formation of a complex between bicarbonate transport proteins and other CA isoforms. In the present report, we examined the relationship between the extracellular CA isoform, CAIV, and plasma membrane chloride/bicarbonate proteins.

Three lines of evidence indicate that CAIV and anion exchangers form a physical complex. GPI-anchored proteins localise to lipid rafts in the plasma membrane (Harder & Simons, 1997). Lipid rafts are areas rich in sphingolipids and cholesterol and are known to remain intact upon cell solubilisation in cold Triton X-100 (Brown & Rose, 1992). It can be assumed that as a GPI-linked protein, CAIV, is localised to lipid rafts. We compared the sedimentation of CAIV in sucrose gradients in the absence and presence of AE1 upon cold solubiliataion in Triton X-100. In the presence of AE1, the sedimentation of CAIV shifted from the less dense fractions, where it is found when expressed alone, and to the denser fractions where AE1 was localised. This result suggests that AE1 and CAIV physically interact and that AE1 pulls CAIV out of lipid rafts. In a second approach, AE1, AE2 and AE3 expressed in HEK 293 cells were able to interact with CAIV from cell lysates of HEK 293 cells expressing CAIV in gel overlay assays.

The third and most definitive evidence of a CAIV/AE interaction came from GST pull-down assays. As CAIV is linked to the extracellular surface of the cell we reasoned that the AE/CAIV interaction occurred at one of the larger extracellular loops of AE1. We investigated the extracellular loops between transmembrane segments 5 and 6 (EC3) and TM 7 and 8 (EC4). GST fusion

proteins of the individual loops (GST-AE1EC3, GST-AE1EC4) were constructed. These GST fusion proteins and control GST alone were immobilised on glutathione Sepharose resin. Lysates prepared from HEK 293 cells transfected with CAIV cDNA or sham-transfected were incubated with the GST protein/glutathione Sepharose resin complexes. CAIV associated with the resin was detected on immunoblots. The presence of a band corresponding to the molecular weight of CAIV appeared only when lysates from CAIV-transfected cells were applied to immobilised GST-AE1EC4 (Figure 6.7). This suggests that CAIV binds specifically to the fourth extracellular loop of AE1.

On the basis of these three lines of evidence we conclude that CAIV forms a complex with AE1, AE2 and AE3. The simplest explanation for our observation is that CAIV directly interacts with AE1, AE2 and AE3. We cannot rule out the possibility that another protein is required to mediate the AE/CAIV interaction. However, the requirement of an intermediary protein is highly unlikely since CAII interacts directly with AE1-AE3 (Sterling et al., 2001) and any intermediary protein would have to be endogenously expressed in HEK 293 cells.

The increase of AE1, AE2 and AE3 bicarbonate transport activity caused by CAIV likely requires a direct interaction between CAIV and AE; localisation of CAIV to the same membrane may not be sufficient to enhance bicarbonate transport rate.

The identification of EC4 as the binding site for CAIV is interesting in a number of ways. Studies of AE1 topology suggest that EC4 is the largest extracellular loop (Reithmeier et al., 1996; Tang et al., 1998), and therefore might

be expected to form an extracellular binding site. That EC4 forms an accessible extracellular region is demonstrated by the four blood group antigens, the Wright antigen (E658K)(Bruce et al., 1995), Mo^a (R656H), Hg^a (R646Q) and Sw^a (R646Q) (Jarolim et al., 1998), which are found in EC4 (Figure 6.6). The Wright antigen is formed by a complex between the highly glycosylated single transmembrane protein glycophorin A, and AE1 (Bruce et al., 1995). Thus there is precedent for an interaction between EC4 and the extracellular moiety of an erythrocyte protein.

A study of the AE1 region from the glycosylation site at N642 (Figure 6.6) through transmembrane segment 8 suggested that the region S643-L655 had a folded structure that was inaccessible to hydrophilic reagents, while the region R656-I661 had an open structure with maximum accessibility at R656 (Tang et al., 1998). Taken together we propose that CAIV interacts with AE1 somewhere in the R656-I661 region. Interestingly this region has been suggested to form the outer vestibule that funnels anions to and from the transport site (Tang et al., 1998). Localisation of CAIV to EC4 would therefore place the enzyme as close as possible to the extracellular aspect of the anion transport site.

The structure of AE2 and AE3 differs from AE1 in that AE2 and AE3 are glycosylated on EC3 rather than EC4 and EC3 is larger than EC4 in AE2 and AE3 (Zolotarev et al., 1996). It is therefore not clear whether AE2 and AE3 interact with CAIV in the homologous loop region or not. Nevertheless, the CAIV-mediated rescue of AE2 and AE3 bicarbonate transport activity in the presence of V143Y CAII indicates a functional interaction between CAIV and AE2 and AE3, which is also likely paralleled by a physical interaction.

We have previously characterised the first example of a transport metabolon by defining the importance of the physical and functional interaction between AEs and CAII (Sterling et al., 2001). The present study provides evidence that the extracellular-anchored enzyme CAIV is the extracellular component of the bicarbonate transport metabolon. The presence of intracellular CAII and extracellular CAIV catalytic activity in the cell and the fact that both enzymes can potentiate the bicarbonate transport activity of AE1 provides the cell with a "push-pull" mechanism for bicarbonate transport (Figure 6.8). CA-mediated production of HCO_3^- on the one side of the membrane will provide "push" for transport by AE and CA-mediated conversion to CO_2 on the other side provides "pull", by minimisation of the [HCO_3^-] at the trans transport side. This push-pull mechanism, established by having CA catalytic activity on both sides of the plasma membrane, accelerates AE-mediated bicarbonate transport as shown in this study.

Although the heart does not express any cytosolic CA, it expresses two extracellular CA isoforms, one of which is known to be CAIV (Sender et al., 1998). The heart also expresses AE1, AE2 and AE3 (Linn et al., 1995; Linn et al., 1992; Pucéat et al., 1995), which were all shown to require interaction with CAII for maximal transport activity to be achieved (Sterling et al., 2001). Our results show that extracellular CAIV can functionally replace CAII. Thus, despite the absence of CAII in cardiomyocytes, these bicarbonate transporters would be expected to be able to function at their maximum rate.

CAIV plays a major role in bicarbonate reabsorption in the kidney (Maren & Conroy, 1993) as well as modulating the pH in the renal lumen

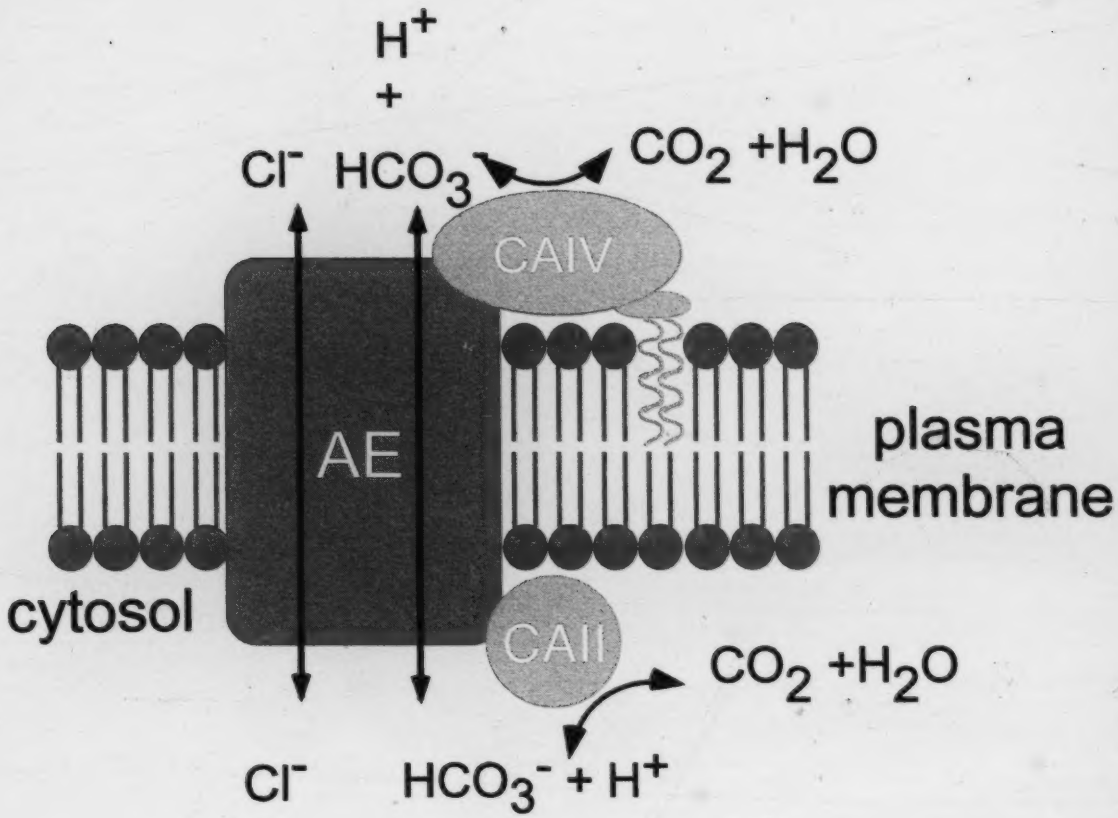

Figure 6.8 A bicarbonate transport metabolon.

Schematic model of the binding of CAIV to extracellular loop 4 of AE1 and CAII to the C-terminal tail, which maximises AE mediated bicarbonate flux by the production and removal of substrate from the transport site. GPI is indicated by the structure that comes off of CAIV and intercalates into lipid bilayer of plasma membrane.

(Brechue et al., 1991). The kidney expresses an N-terminally truncated variant of AE1 (kAE1) (Bagnis et al., 2001). Although it is generally agreed that kAE1 localises to the basolateral surface of α-intercalated cells, there is also one report that kAE1 is found at the apical surface of β-intercalated cells, where it could interact with CAIV (van Adelsberg et al., 1993). AE2 is found in the basolateral surface of many portions of the kidney (Brosius-III et al., 1995). CAIV has been reported to be in both apical and basolateral surfaces of proximal tubule (Schwartz et al., 2000) (Schwartz et al., 1999) and basolateral surface of thick ascending limb (Brown, Zhu & Sly, 1990), but others report that CAIV is only found apically in the kidney (Lonnerholm & Wistrand, 1991). Therefore AE1 and AE2 co-localise with CAIV in some renal cells.

CAII deficiency is an autosomal recessive condition characterised by renal tubular acidosis and osteopetrosis (Sly et al., 1983). Despite gross abnormalities associated with the absence of CAII, the CA activity in erythrocytes appears to be adequate and patients do not exhibit abnormalities in CO_2 transport (Dodgson et al., 1988). The findings in the present paper, along with the recent detection of CAIV expression and activity in human erythrocytes (Wistrand et al., 1999) explain the ability of erythrocytes lacking CAII to sufficiently accommodate CO_2 metabolism. We found that the loss of AE activity induced by the dominant-negative V143Y CAII could be rescued by CAIV. This reconciles the normal CO_2 carrying capacity of blood in CAII-deficient patients.

Normal bone reabsorption requires basolateral AE2 in osteoclasts. CAII deficiency decreases AE2 activity which results in osteopetrosis. CAIV is absent from osteoclasts and therefore cannot compensate for the lack of CAII activity in

CAII deficiency. Renal tubular acidosis results from a failure to reabsorb bicarbonate from the renal tubular lumen. It is possibile that CAIV and AE proteins can interact in the kidney, however, the presence of functional CAIV in CAII deficient patients is not sufficient to prevent renal tubular acidosis.

This study presents several lines of evidence indicating a physical interaction of extracellular CAIV with the Cl^-/HCO_3^- transport protein. We have also demonstrated that the presence CAIV catalytic activity accelerates the movement of bicarbonate across the plasma membrane by AE1, AE2 and AE3. The results described here demonstrate that CAIV is the extracellular component of a bicarbonate transport metabolon, formed along with an anion exchange protein and intracellular CAII. When co-expressed, CAII and CAIV contribute to the function of AE, providing a "push-pull" mechanism for bicarbonate movement across the plasma membrane. The potentiation of AE transport activity by CAIV in the erythrocyte may provide an explanation for the observation that CAII deficient patients retain the capacity for normal CO_2 metabolism and transport. It also suggests a potential mode of regulation of bicarbonate transport and raises the possibility that modulation of CAIV/AE interaction, perhaps with antibodies or peptides, could be a viable therapeutic approach to alter bicarbonate transport.

Chapter 7

Summary and future directions

Summary and Future Directions

Although at the time I began the studies presented in this thesis the role that the AE family of Cl^-/HCO_3^- anion exchange proteins played in regulation of pH_i was plainly important, it was not however clearly understood. As outlined throughout the thesis, maintenance of pH_i is paramount to preservation of normal cellular function. This underscores the need to discern the role of AE proteins in pH_i. The presence of different, and in some cases multiple, isoforms of AE proteins in various tissues makes the role of AE proteins even more complicated. An understanding of the expression, function and regulation of each individual isoform is therefore important to determine the contribution of each isoform to maintenance or recovery of pH_i.

7.1 Regulation of Cl^-/HCO_3^- transport activity of AE proteins by pH

The studies outlined in **chapter 3** were designed to determine the potential contribution of each AE isoform to regulation of pH_i. HEK 293 cells were transiently transfected with individual AE isoforms and transport activity was monitored by following changes in intracellular pH associated with AE-mediated Cl^-/HCO_3^- transport. AE1 and AE2 had a transport activity that was approximately 8 times that of full-length AE3 and AE3c.

During an ischaemic episode cells produce acid as a byproduct of metabolism and pH_i can reach values as low as pH 6.2 (Orchard & Kentish, 1990). Some of the acid is removed as lactate by MCT (Poole & Halestrap,

1990). Some of the acid is removed as lactate by MCT (Poole & Halestrap, 1993) and H^+ that are removed from the cell accumulate in the interstitial fluid due to reduced or absent perfusion. Thus, both intracellular and extracellular fluids become acidic.

By clamping pH_i and pH_o at the same value we could mimic the conditions of an ischaemic episode and investigate the impact of changes in pH_i/pH_o on AE transport activity. Using SPQ, we monitored AE mediated Cl^- transport while clamping pH_i/pH_o at set values and determined that while AE2 was steeply negatively regulated by changes in pH_i/pH_o, AE1 and AE3 were insensitive to changes in pH_i/pH_o in the range pH 6.0-9.0. These results suggest that both AE1 and AE3 could play a role in recovery of pH_i following an ischaemic episode.

7.2 CAII binds to the C-terminal tail of AE1, but not DRA to regulate AE-mediated transport activity

The carbonic anhydrases are a family of metalloenzymes involved in the hydration/dehydration of CO_2/HCO_3^-. The hydration of CO_2 by CA produces HCO_3^-, the substrate for AE and thus contributes to AE transport. Following flux of HCO_3^-, the dehydrating action of CAII reduces the localised HCO_3^-concentration, thus maintaining the HCO_3^- gradient across the plasma membrane. Together, CAII and AE proteins contribute to the regulation of pHi and remove the metabolic waste product, CO_2, from the body.

Prior to our investigations, several lines of evidence had shown an interaction between CAII and AE1. Binding of erythrocyte membranes to CAII increased CAII enzymatic activity (Parkes & Coleman, 1989) and reaction of DIDS with AE1 altered the binding of a fluorescent inhibitor to

carbonic anhydrase (Kifor et al., 1993). CAII was noted to distribute to the plasma membrane in erythrocytes and an extracellular lectin caused agglutination of AE1 and a similar redistribution of CAII on the cytosolic surface of the erythrocyte membrane (Vince & Reithmeier, 1998), suggesting a physical interaction of AE1 with CAII. Co-immunoprecipitation and a binding assay showed that CAII but not CAI interacts with the C-terminus of AE1 (Vince & Reithmeier, 1998). Truncation and point mutation of the AE1 C-terminus, identified the binding site of CAII in human AE1 as LDADD (amino acids 886-890) (Vince & Reithmeier, 2000) and showed that the basic N-terminal region of CAII forms the AE1 binding site (Vince et al., 2000).

The study outlined in **chapter 4** was aimed at determining the functional impact of the CAII/AE interaction. Incubation with acetazolamide, a CA inhibitor, reduced AE transport activity by up to 70% while subjecting cells to the same assay in the absence of acetazolamide had no effect on transport activity. To date acetazolamide is the only CA inhibitor characterized that does not directly inhibit AE-mediated Cl^-/Cl^- self exchange at a 5 mM concentration. We found a maximum inhibition of AE-mediated Cl^-/HCO_3^- exchange with 250μM acetazolamide (Cousinn & Motais, 1975a, 1975b) and therefore conclude that the effect on AE occurred indirectly via inhibition of CAII. Mutation of the CAII binding site from DADD to either NANN or AAAA resulted in loss of CAII binding (Vince & Reithmeier, 2000) and AE-mediated transport activity. An over-expressed functionally inactive CAII mutant, V143Y, can compete with endogenous CAII for the AE binding site. Displacing active CAII from the C-terminus of AE1, AE2 and AE3 reduced AE transport activity. Taken together, these results indicate that the

physical interaction of CAII with AE potentiates AE-mediated bicarbonate flux.

The CAII/AE interaction has been likened to a transport metabolon, a physical complex of proteins linking a cytosolic enzyme to a transporter. This raises the question of whether the metabolon increases AE transport activity via a kinetic effect or an allosteric one. Several pieces of evidence point to a kinetic effect. Of the two non-binding mutants one retained full functional acivity as assessed by Cl^-/Cl^- self exchange (Seth Alper, unpublished observation). This indicates that although this AE protein cannot bind CAII, the anion exchange capacity of the protein is still intact even though it's HCO_3^- transport capacity is severly impaired. The binding of V143Y CAII to the C-terminal tail of AE proteins also impaired transport activity. Should the AE/CA interaction have a n allosteric effect on AE transport activity it would be difficult to see an effect on transport activity in the dominant neative experiment. Therefore, the fact that AE retains anion exchange transprt function upon loss of the CAII binding site along with the results from the dominant negative experiments indicates that the AE/CAII interaction maximises AE transport activity kinetically as opposed to by a conformational change.

It has however been recently noted that interaction of CAII with the binding motif peptide of AE1 (LDADD) increases CAII activity (Scozzafava & Supuran, 2002). These results indicate the possibility of an allosteric effect occurring with CAII as opposed to AE1 upon AE/CAII interaction. Therefore maximizing AE transport activity by the interaction between AE1 and CAII may also be in part due to increasing CAII turnover rate.

Modulation of the CAII/AE interaction would provide a cell with a mechanism to regulate AE transport activity and thus movement of HCO_3^- into and out of the cell. Regulating HCO_3^- flux in this manner would provide a means to regulate pH_i.

Comparison of the C-terminal tail of other bicarbonate transport proteins revealed that, with the exception of DRA, all of the bicarbonate transport proteins investigated contained a putative CAII binding site. Results presented in **chapter 5** show that over-expression of V143Y CAII had no effect on DRA-mediated HCO_3^- transport activity indicating that DRA and CAII do not form a transport metabolon. Therefore, it is feasible to suggest that DRA-mediated HCO_3^- transport activity is regulated by some other means than AE-mediated transport activity.

7.3 CAIV binds to the fourth extracellular loop of AE1 and regulates AE-mediated transport activity

The results in **chapter 4** indicate that AE proteins require binding of CAII at their C-terminus to accelerate transport activity. The absence of cytosolic CA in cardiomyocytes would therefore suggest that AE transport proteins do not function at their maximum capacity in cardiomyocytes. Only an extracellular CA, CAIV, had been reported in cardiomyocytes, therefore we investigated the possibility of an interaction of AE with CAIV.

Chapter 6 documents a series of studies that demonstrated a functional and physical interaction between AE proteins and CAIV. Over-expression of V143Y reduces the effect of CAII binding on AE-mediated bicarbonate transport. Expression of CAIV, in the presence of V143Y, rescued the decreased AE transport activity, indicating a functional interaction between

AE proteins and CAIV. Co-migration on sucrose gradients and overlay assays demonstrated a physical interaction between AE1, AE2 and AE3. GST pull-down assays localised the AE1/CAIV interaction to the fourth extracellular loop of AE1. These results suggest that CAIV can form the extracellular component of the bicarbonate transport metabolon and interaction between AE transport proteins and CAIV maximises AE-mediated HCO_3^- transport.

7.4 Future Directions

To delineate every factor that regulates a family of proteins is a monumental task. However determining some basic regulatory mechanisms can make great strides towards understanding the contribution of a protein to certain circumstances.

We have started a wide screen of the effect of second messenger pathway stimulating factors on AE transport activity. By performing this screen on individual AE isoforms expressed in HEK 293 we can narrow in on some of the pathways that when stimulated had an effect on AE transport activity. This will help to clarify the possible modes of regulation present in the much more complicated cardiomyocyte. Identification of the intracellular signaling pathways responsible for AE activation will point towards potential regulatory mechanisms. For example, the tyrosine phosphatase inhibitor, orthovanadate, had a negative impact on both AE3 and AE3c transport suggesting that tyrosine kinases inhibit AE3 activity isoforms activity (D. Sterling and J. Casey, unpublished observation). By establishing the kinase activity responsible for impairing AE3 transport activity, the impact of this

kinase activity *in vivo* can be investigated and mechanisms to block AE3 inhibition instituted.

The ever-expanding field of transgenics mouse models begs to be used as a tool to delineate the contribution of each AE isoform to pH_i regulation in cardiomyocytes. AE knock-out mice, especially tissue specific knock-outs, will provide excellent experimental models for future investigation into the impact of each AE isoform on many physiological processes.

We established the physical and functional interaction between AE proteins and CAII/IV. Definition of the transport metabolon however is only the tip of the iceberg and many factors regarding these interactions remain to be studied. Ongoing work in the lab has detected a similar interaction between the NBCs and CA proteins (Alvarez, Loiselle and Casey, manuscripts in preparation). The number of bicarbonate transport proteins identified is growing rapidly and out of all the proteins tested so far we have noted only one exception to the rule, DRA. It would seem then that DRA does not directly interact with CAII. It remains to be determined if an intermediary protein brings CAII into close enough proximity to DRA to accelerate DRA-mediated HCO_3^- flux. Alternately DRA-mediated HCO_3^- flux may be regulated by alternative means. This study also warrants investigation into the formation of transport metabolons between other kinds of transport proteins and enzymes, not just HCO_3^- transport proteins.

Another area of interest is determining factors that can regulate the interaction between AE and CA. By removing CAII from its binding site on AE proteins the flux of HCO_3^- across the plasma membrane will be greatly reduced. Conversely by promoting the AE/CA interaction HCO_3^- flux will be

maximised. Therefore mechanisms that regulate the AE/CA interaction provide a cell with a fast, effective method for tight regulation of pH_i by manipulating AE transport activity.

Clearly there are still many areas of research to be investigated before we can clearly understand the role that AE proteins play in regulating pH_i. Identification of some of the regulatory mechanisms that impact AE expression and transport activity will help clarify the possible modes of regulation of AE transport and provide a basis for potential therapeutic manipulation of AE proteins.

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